

1971

Pathogenesis Of Chemically Induced Malignant Lymphoma In Mice

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PATHOGENESIS OF CHEMICALLY INDUCED MALIGNANT LYMPHOMA
IN MICE

by

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada

July 1970

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The author is grateful to the
Medical Research Council and National
Cancer Institute of Canada for providing
financial support for this study.

ACKNOWLEDGEMENTS

I extend my most sincere thanks to Dr. J. V. Frei for the guidance and help he gave to carry out this study and for the understanding and consideration he showed on many occasions during the last two years.

The author is grateful to Dr. J. A. McCarter and Dr. Judith K. Ball for the guidance and help they gave in carrying out this study. The constructive criticisms and helpful suggestions of other members of the department and fellow students are gratefully acknowledged.

Sincere thanks are extended to Mrs. P. Edgeworth, Mrs. N. Estick, Mr. B. Forbes, Mrs. S. Hoshino, Mrs. D. Moore and Mrs. J. Oliver for technical assistance.

I am indebted to Dr. A. C. Wallace and Dr. M. Daria Haust for the encouragement and help they gave during the period of graduate training.

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ABSTRACT

The pathogenesis of chemically induced malignant lymphoma in mice was studied.

The morphological changes in the lymphoreticular system during the development of malignant lymphoma induced by methylnitrosourea (MNUA) could be divided into 3 stages, viz., cell depletion, regeneration, and neoplasia. Although the thymus was the site of origin of malignant lymphomas, the bone marrow, spleen, and lymph nodes were also affected during the genesis of malignant lymphoma. The evolution of the neoplastic changes in the thymus was described in detail, and at least in some instances, malignant lymphoma involving the thymus arose by confluence of more than one focus of neoplasia. The evidence suggested that the involvement of other lymphoid organs occurred by metastasis from the thymic lymphoma. Most malignant lymphomas were lymphoblastic and many had a starry-sky pattern due to the presence of large periodic acid-Schiff (PAS) positive cells, around which the tumour cells showed mitotic activity. Lymphoid follicles formed in the medulla of the thymus during the genesis of malignant

lymphoma. The possibilities that a virus may be implicated in the genesis of MNUA-induced lymphoma and that immunological reactions may be part of the development are discussed.

The study of dose-response relationships during the development of MNUA-induced malignant lymphoma showed that in the single dose group, early mortality was first seen at 75 mg/kg, the highest incidence of malignant lymphoma was 72%, and the shortest latent period was 144 days. In the fractionated dose group, the pattern of fractionation, i.e., the interval between the fractions, influenced all 3 parameters. In the daily fractionated group, early mortality first appeared at the total dose of 200 mg/kg, the highest incidence of malignant lymphoma was 93%, and the shortest latent period was 139 days. In the weekly fractionated group, early mortality first appeared at the total dose of 250 mg/kg, the highest incidence of malignant lymphoma was 93%, and the shortest latent period was 87 days. Thus reduction of early mortality, enhancement of incidence, and shortening of latent period of malignant lymphoma were the main effects of fractionated doses of MNUA. Although the precise mechanism of these effects is unknown, multiple stages may be involved in the genesis of MNUA-induced malignant lymphomas.

The study of transplantability of thymus cells during the development of malignant lymphoma induced by

7,12-dimethylbenz [a] anthracene (DMBA) showed that the thymus cells become transplantable at 35 days after treatment. Most of the malignant lymphomas occurring after transplantation of early neoplastic cells showed exclusive or predominant involvement of thymus and no bone marrow involvement. On the other hand the malignant lymphomas occurring after transplantation of full-fledged neoplastic cells showed widespread involvement of the lymphoreticular system and of the parenchymatous organs with a leukaemic blood picture. That the difference in the distribution may be related to different numbers of cells injected cannot be completely ruled out. However, the distribution of malignant lymphoma may be indicative of the sites of differential attachment and proliferation, or "homing" capacity of early and full-fledged neoplastic cells and of the phenomenon of tumour progression.

I. INTRODUCTION

The title of the thesis is "Pathogenesis of chemically-induced malignant lymphoma in mice." Pathogenesis, defined as "the course of development of disease, including the sequence of processes or events from inception to the characteristic lesion or disease" (Blakistone's New Gould Medical Dictionary 1965), was studied from three points of view: the first dealing with morphological aspects, the second dealing with dose response relationships and the third dealing with transplantability of thymus cells during the development of chemically-induced malignant lymphomas in mice. Methylnitrosourea (MNUA) was used as the inducing agent of malignant lymphoma to study the first two aspects and 7,12-dimethylbenz [a] anthracene (DMBA) to study the third. This last inducing agent was used because preliminary data regarding the transplantability of thymus cells during the course of development of DMBA-induced malignant lymphoma were available in this laboratory as a result of preliminary observations made by Dr. Judith K. Ball.

Most workers in the field of research use the terms leukaemia and malignant lymphoma interchangeably. Leukaemia and malignant lymphoma are interrelated and at times overlapping diseases. Similar cell types may be involved in the two disease processes, e.g. small lymphocyte in chronic lymphocytic leukaemia and in malignant lymphoma of well-differentiated lymphocytic type; lymphoblast in acute lymphoblastic leukaemia and in malignant lymphoma of stem cell type (Lukes, 1968). The differences are in the distribution of neoplasia in typical examples of each and also in man in clinical presentation and in the approach to therapy. In leukaemia there is diffuse involvement of the bone marrow by specific types of neoplastic cellular proliferation which is typically associated with uniform involvement of spleen, liver and lymph nodes. The distribution of malignant lymphomas by contrast is irregular and variable and may be limited to a single group of lymph nodes or be a single mass. The bone marrow, peripheral blood, spleen and liver may be involved, or may exhibit irregular nodular involvement (Lukes, 1968). The terms leukaemia and malignant lymphoma for the purpose of uniformity and definition of typical examples of each should be used on the basis of these differences, recognizing at the same time the inter-relationship and the occasional overlap of the two disease processes. The literature on spontaneous and induced

neoplasms of the reticular system of the mouse (Frei, 1970; Gardner, Dougherty and Williams, 1944; Kaplan, 1967; Metcalf, 1966; Moloney, 1959; Rappaport and Baroni, 1962) shows that because of the origin of these tumours in the thymus, and because of a lack of uniform and consistent involvement of the bone marrow and other reticular organs, these tumours should be designated as malignant lymphomas. Whenever diffuse involvement of the bone marrow with leukaemic blood picture occurs it should be referred to as a leukaemic transformation or a leukaemic phase of malignant lymphoma. This terminology will therefore be used in this thesis.

II. REVIEW OF LITERATURE

To avoid repetition, the pertinent literature on the pathogenesis of malignant lymphoma in mice with respect to morphological changes, dose response relationships and transplantability will be reviewed when the results of the appropriate experiments are discussed. The literature on other aspects of malignant lymphoma pertinent to this project is reviewed below.

a) Historical Aspects

Hodgkin's disease (though not under that name) was the first among the neoplastic lesions of the haemato-poietic system to be recognized in man. The morbid anatomical appearances of lymph nodes and spleen in this disease were first described by Hodgkin (1832). First cases of leukaemia were reported by Craigie and Bennett (1845) and by Virchow (1846). Craigie and Bennett (1845) attributed the remarkable numbers of white blood cells to the presence of "purulent matter in the blood." Virchow (1846) who introduced the term leukaemia, considered that the haematological changes were part of a definite pathological process

involving certain organs of the body. As more human cases were studied the complexity of leukaemia became evident. Virchow (1846) recognized two forms of leukaemia. In one, the small forms of white cells predominated and enlargement of lymph nodes was common; in the second, the large white cells increased in number and splenomegaly occurred. After myelopoietic functions of the bone marrow were recognized (Neumann, 1870) myeloid leukaemia as a disease entity was established. Kundraat (1893) recognized lymphosarcoma (malignant lymphoma) as a disease entity different from leukaemia. Acute leukaemia was first described by Friedrich (1857).

The earliest report of murine lymphoma was by Eberth (1878). He noted an enormous spleen in a mouse. Histologically the spleen and the liver were infiltrated with cells resembling lymphocytes. Haaland (1905) gave the best of the early descriptions noting the excessive enlargement of lymph nodes, a mediastinal tumour and infiltration of liver, kidney and spleen.

b) Spontaneous Lymphoma in Mice

AKR and C58 are the two strains of mice produced by selective inbreeding, which have an over 90% incidence of spontaneous lymphoma of the lymphoblastic type (Potter and Richter, 1932; Metcalf, 1966).

Potter, Victor and Ward (1943) who studied the histological changes preceding the development of spontaneous lymphoma in the C58 strain found that the initial neoplastic changes were seen in the lymph nodes and in periportal areas of the liver. In later stages the disease was widespread. The authors did not mention the thymus in their paper. In AKR lymphoma the thymus is the site of origin and the peripheral blood may or may not show increased numbers of abnormal cells (Metcalf, 1966). In mice of the SJL/J strain spontaneous lymphoma is of the reticulum cell type and originates in the mesenteric lymph nodes and in Peyer's patches (Siegler and Rich, 1968).

c) Induced Lymphoma in Mice

Lymphoma can be induced in mice by various agents used either singly or in combination.

1) Radiation: The first successful induction of malignant lymphoma resulted from irradiation (Krebs et al., 1930). Kaplan (1966) has extensively studied radiation-induced lymphoma. It is thymic in origin and also involves the lymph nodes, spleen, liver and other parenchymatous organs.

2) Viruses: Gross (1951) inoculated a group of less than 12 hours old C3H mice with a cell-free filtrate prepared from lymphomatous tissues of AKR mice with spontaneous

or transplanted lymphoma. Within 6 to 9 months 7 of the 25 inoculated mice developed lymphoma whereas the incidence of lymphoma in the controls was less than half per cent. Subsequently Gross (1957) developed a potent stable strain of the virus (Passage A) by repeated cell-free passage in susceptible mice permitting the confirmation of viral aetiology of murine lymphoma in several laboratories. In the same year Charlotte Friend (1957) reported the development of a biphasic disease following the inoculation of a Swiss strain with cell-free extracts of Ehrlich ascites carcinoma. This biphasic disease was characterized by splenic enlargement. The first phase occurring between 10 and 30 days after inoculation of the virus was characterized by marked splenic enlargement due to diffuse proliferation of erythroid series of cells. The second phase occurred between 50 and 80 days and was characterized by splenic enlargement due to diffuse proliferation of "reticulum cells" and there was peripheral lymphocytosis; the thymus and lymph nodes were apparently not involved (Metcalf, Furth and Buffett, 1959; and Siegler, 1968). Rauscher (1962) isolated a potent virus from splenic tissues of BALB mice. The disease resulting from inoculation of the virus was also biphasic. In the first phase which occurred between 25 and 35 days the spleen was enormously enlarged due to diffuse proliferation of erythroid cells. There was no

thymic involvement. In the second phase which occurred between 60 to more than 90 days the spleen, lymph nodes and thymus were enlarged due to proliferation of lymphoid cells. The possibility of there being two viruses, each of which is responsible for a separate phase of the bi-phasic disease could not be supported by electron microscopic or serial passage studies. This was followed by other reports of lymphoma induced in mice upon inoculation of cell-free extracts prepared from a transplantable mouse sarcoma by Moloney (1959) and from an X ray induced lymphoma of C57 Black mice by Lieberman and Kaplan (1959). At the present time more than 15 reports on the induction of lymphomas by cell-free materials from a variety of sources have appeared in the literature (Rich and Siegler, 1967; Rich, 1968).

3) Chemicals: Kirschbaum, Strong and Gardner (1940) studied malignant lymphomas induced by 3-methylcholanthrene. Fiore-Donati et al. (1961) reported induction of malignant lymphomas in newborn mice by urethane. The lymphomas were thymic in origin. Rappaport and Baroni (1962) studied the pathogenesis of 7,12-dimethylbenz [a] anthracene (DMBA)-induced lymphomas in mice. These lymphomas were thymic in origin and mostly of stem cell type. More recently methyl-nitrosourea has been shown to induce malignant lymphomas in

new-born and adult mice (Graffi and Hoffmann, 1966; Terracini and Stramigoni, 1967; Kelly, O'Gard, Yancey and Botkin, 1968; Frei, 1970).

4) Hormones: Lymphomas induced by oestrogenic hormones have been described by Gardner et al. (1944). The thymus was the site of origin with dissemination to other lymphoid tissues. In some animals abnormal cells were seen in increased numbers in the peripheral blood. The lymphomas were lymphoblastic in type. Law (1947) reported an increased incidence of spontaneous lymphoma in C58 mice by orchidectomy in mature males or adrenalectomy in immature males and females. On the other hand incidence of spontaneous and radiation-induced lymphoma was decreased by administration of cortisone (Upton and Furth, 1954).

5) Viruses in chemically and hormonally induced malignant lymphomas: Several reports have appeared in the literature indicating that cell-free extracts prepared from chemically induced lymphomas in mice (Irino, Ota, Sezaki and Zuzaki, 1963; Toth, 1963; Ribachi and Giraldo, 1966; Haran-Ghera, 1967) and possibly even oestrogen induced lymphomas (Kunii, Takemoto and Furth, 1965) induce lymphomas in the recipients. This evidence suggests that strains of mice with a low incidence of spontaneous lymphoma harbour a latent virus and that exposure to various physical or

chemical agents results in a change in the host-virus relationship leading to development of lymphoma.

6) Combination of agents inducing lymphomas: The effects of combination of leukaemogenic agents were investigated by Kirschbaum and Mixer (1947). Using ionizing radiations, carcinogenic hydrocarbons and oestrogenic hormones they induced leukaemia in 8 inbred strains of mice which varied in susceptibility to the development of spontaneous lymphoma. Kirschbaum et al. (1953) investigated the effects of three leukaemogens--X rays, oestrogenic hormones and methylcholanthrene--on the strains BALB/c, CBA and DBA. In BALB/c and CBA mice X rays and oestrogenic hormones acted synergistically while methylcholanthrene was ineffective. In DBA mice oestrogenic hormones enhanced the onigenic potency of methylcholanthrene and X rays. All the neoplasms were lymphocytic type and most showed predominant involvement of the thymus. Other workers (McEndy, Boon and Furth, 1942; Toth, Rappaport and Shubik, 1962; Nishizuka and Slusa, 1968) have also demonstrated the synergistic effects of combination of lymphoma-inducing agents such as X rays, methylcholanthrene, DMBA and hormones.

d) Carcinogenesis by Nitroso Compounds

Magee and Barnes (1956) first demonstrated the carcinogenic action of dimethylnitrosamine. Since then about 40

different compounds have been shown to be carcinogenic in various species of animals (Magee and Barnes, 1967). Methylnitrosourea (MNUA) induces tumours of the stomach, lung, intestine and kidney in mice and rats (Schoenthal, 1963; Magee and Barnes, 1967). Graffi and Hoffmann (1966) first reported induction of lymphomas in mice by using MNUA. This was followed by other reports of MNUA-induced malignant lymphoma in mice (Terracini and Stramigoni, 1967; Kelly et al., 1968; and Frei, 1970). Graffi and Hoffmann (1966) induced malignant lymphomas in new-born mice by application of MNUA (the exact method of application is not described by authors). The tumours involved the lymph nodes, spleen, liver and other parenchymatous organs. The total incidence was 48.8%. Terracini and Stramigoni (1967) induced malignant lymphomas in new-born mice by giving MNUA subcutaneously. The tumours involved the thymus, lymph nodes, spleen, bone marrow and parenchymatous organs. The total incidence was 75%. They also succeeded in inducing malignant lymphomas in adult mice but the experiment was incomplete at the time of reporting. Kelly et al. (1968) obtained 30 to 80% incidence of lymphomas in mice given MNUA intracerebrally or subcutaneously. The tumour appeared to originate in the thymus and also involved other lymphoid and parenchymatous organs. Frei (1970) reported 63 to 72% incidence of lymphomas in new-born and adult

inbred Swiss mice given MNUA intraperitoneally. Most of these tumours predominantly involved the thymus.

e) Common Denominators in Spontaneous and Induced Murine Lymphomas

Most of the spontaneous (AKR) and induced (radiation, DMBA, methylcholanthrene, MNUA, oestrogens, virus) murine lymphomas have the following common characteristics: an origin in the thymus with exclusive or predominant involvement of the thymus; the possibility of interaction of the virus and the respective inducing agent used; the lymphoblast or lymphocyte as the predominant tumour cell type; and the occurrence of necrosis and cell depletion in the reticular system in the initial stages (Metcalf, 1966; Siegler and Rich, 1968; Rappaport and Baroni, 1962; Kirschbaum et al., 1940; Frei, 1970; Gardner et al., 1944; Levinthal, Buffett and Furth, 1961; Irino et al., 1963; Toth, 1963; Kunii et al., 1965; Ribuchi and Giraldo, 1966; Haran-Ghera, 1967). A systematic study of bone marrow has not been done in most of these tumours but from the descriptions it appears that the bone marrow and the peripheral blood are not consistently involved in the disease process.

f) Involvement of the Thymus in Leukaemia and Malignant Lymphoma in Man

Text-books and monographs scarcely mention thymic involvement in leukaemias and malignant lymphomas. Nevertheless, thymic enlargement in leukaemias and lymphomas including Hodgkin's disease occurs more frequently than is generally recognized. Histological evidence of infiltration of the thymus is quite commonly seen in these neoplastic disorders. Thus Middleton (1966) described it in 18 of the 42 cases he examined. In addition there are numerous reports in the literature describing marked thymic enlargement in leukaemias and lymphomas (Bichel, 1947; Cook, 1932; Davis and McGreadis, 1962; Gilmartin, 1963; Katz and Lattes, 1969; Lattes, 1962; Patey, 1963; Thompson, 1955; Webster, 1961). In some patients thymic enlargement precedes leukaemic blood picture or is the first manifestation of lymphoma (Bichel, 1947; Cook, 1932). Bichel (1947) suggested that thymic enlargement producing pressure symptoms with concomitant or subsequent development of leukaemic blood picture should be considered as a distinct clinical syndrome. Most of the cases of leukaemia with involvement of the thymus were of the acute lymphatic or undifferentiated type. However Gilmartin (1963) reported the occurrence of thymic enlargement in patients of acute myeloid leukaemia, chloroma and acute monocytic leukaemia. Cook (1932) reviewed cases of thymic involvement in leukaemia and found

that it was 6 times as common in males as in females and 90% of the cases occurred below the age of 30 years.

Webster (1961) noted that patients with lymphoma with involvement of thymus are more prone to develop an acute leukaemic phase than patients with lymphoma without thymic involvement. Simmon (1950) postulated on the basis of cases reported in the literature and on the evidence in experimental leukaemias and lymphomas, that leukaemia in man may be thymic in origin. Thompson (1955) reviewed the clinical and pathological features of 275 cases of Hodgkin's disease and found that in 112 cases there was thymic enlargement with histological evidence of Hodgkin's disease in the thymus. These findings, other reports in literature, and the similarity of cellular features of Hodgkin's disease to a developing thymus suggested to him that Hodgkin's disease originates in the thymus. Lattes (1962) reported 7 cases of "granulomatous thymoma." However Katz and Lattes (1969) re-evaluated these and 24 additional cases and concluded that these tumours represent a peculiar manifestation of Hodgkin's disease involving the thymus.

As described, the thymus is the site of origin of murine lymphomas and leukaemias. Further, thymectomy has been shown to prevent development of spontaneous murine lymphoma (Law and Miller, 1950), of lymphoma induced by viruses (Miller, 1960), by carcinogens (Law and Miller, 1950), or by X-irradiation (Kaplan, Brown and Paull, 1953).

Thymus grafts restore the susceptibility to lymphoma (Miller, 1962). In view of the evidence in experimental lymphomas and leukaemias, and of the observations in human disease, it is not surprising that thymectomy has been attempted as a form of treatment of lymphoma and leukaemia in man. Earle et al. (1951) reported that thymectomy failed to influence the course of acute and subacute lymphocytic leukaemia in four children. Jiji, Sacks, Linberg and Spurling (1965) performed thymectomy in 3 patients with acute leukaemia during drug-induced remission, but this failed to influence the course of the disease. Gotoff (1968) referred briefly to the case of a male infant treated at 68 hours of life by total thymectomy for neonatal promyelocytic leukaemia; at the age of 2½ years there was no physical abnormality, no evidence of immune deficiency or of leukaemia.

The failure of thymectomy to influence the course of leukaemia in most instances is probably due to the fact that clinically apparent enlargement of the thymus in man represents a late stage of the disease process.

Patey (1963) performed thymectomy in 5 patients of Hodgkin's disease. Histologic evidence of Hodgkin's disease was found in the thymus of 2 patients. All 5 patients were alive 6 years after thymectomy although 2 had developed recurrent manifestations of the disease and 1 developed myeloid leukaemia.

III. EXPERIMENTAL WORK

A. Sequential morphological changes in the lymphoreticular system during genesis of malignant lymphoma induced by a single injection of methylnitrosourea (MNUA)

1. Material and Methods

Mice: Six to seven week old male CFW/D mice (Ball, Huh and McCarter, 1964) originally obtained from Carworth Farms, New City, New Jersey, and inbred by strict brother with sister mating for more than 50 generations in this laboratory were used. They were housed in groups of six in plastic cages with stainless steel tops. The bedding was sterilized sawdust. Water and Purina laboratory chow were freely available.

Carcinogen treatment: The carcinogen, MNUA, was obtained from K & K Laboratories, Plainview, N.Y. The purity of the compound is 85%, with the melting point of 123-126°C.* It was dissolved in cold Standard Saline

*Data obtained from K and K Laboratories.

Citrate (SSC:0.15M Sodium chloride, 0.015M Sodium citrate). The solution was made immediately prior to the treatment and injected intraperitoneally within one hour after the drug had begun to dissolve. The half-life of MNUA dissolved in SSC has been shown to be 3.5 hours at 20°C. Thus not more than 18% of MNUA was destroyed when it was dissolved in SSC and injected within one hour (Frei, 1970). Because of the photosensitivity of MNUA (Magee and Barnes, 1967) the preparation of solutions and injection were done in subdued light. A dose of 75 mg MNUA/Kg body weight was given. This dose produces a 63 to 72% incidence of malignant lymphoma (Frei, 1970). The mice were weighed to the nearest gram.

Serial sampling: Ten male mice from the experimental group were killed by cervical dislocation at days 0, 1, 5, 10, 15, 20, 30, 40 and then every 20 days up to 200 days after injection of MNUA. These time intervals were chosen on the basis of previous studies (Frei, 1970). Ten male mice of the same age from a control group which were given no treatment were killed each time at day 0, 30, 50, 100, 150 and 200 days after injection of MNUA to the experimental group. The animals were allocated in a random fashion at each time interval. Those animals that died during the course of the experiment were excluded from the study.

They were autopsied and separate records of their autopsies were kept.

Parameters studied: Weights of the thymus and spleen were noted. Histological study was done of the thymus, spleen and mesenteric lymph node at each time interval. Differences in weight between control and experimental organs were tested using the Student's t test. In animals having grossly detectable malignant lymphomas, bone marrow from the femoral and tibial bones, peripheral lymph nodes, liver, kidneys and lungs were also studied histologically. All tissues were fixed in Bouin's solution and 3 microns thick sections were stained with haemotoxylin and eosin (H & E). Sections of the thymus were also stained with periodic-acid-Schiff (PAS). Fifteen to 30 sections of thymus were studied. For statistical evaluation of association of tumour cell mitoses with the PAS positive cells seen in the thymic lymphomas, at least 100 microscopic fields of 15x15 microns were studied. These microscopic fields were divided into four categories--those containing PAS positive cell but no mitotic tumour cells; PAS positive cells and mitotic tumour cells; mitotic tumour cells but no PAS positive cells and no mitotic tumour cells or PAS positive cells. χ^2 test with Yates correction was done on the data obtained in 27 of the 44 tumours. In the remaining 17 tumours χ^2 test could not be done on the data for lack of

sufficient number of mitoses or PAS cells in a reasonable number of microscopic fields. Imprints of thymic malignant lymphomas and normal thymuses were made and stained with Wright's stain.

Total and differential white cell counts were done on all animals and absolute neutrophil and lymphocyte counts were calculated. Differences between control and experimental values were tested using the Student's t test.

2. Results

Thymus: The changes in the weight of the thymus after treatment with MNUA are recorded in Fig. 1. It was observed that there was a rapid and significant fall in the weight of the thymus starting at one day after treatment continuing up to day 5. This was followed by gradual recovery with return to normal level at day 30. Between day 30 and 80 the thymus weights of the experimental group were about the same as the control group (fig. 1). The difference in the mean weights at day 60 and day 80 was not statistically significant. From day 100 on, one or more gross thymic malignant lymphomas were observed in the experimental group which resulted in marked scatter in the thymus weights.

The microscopic changes in the thymus after treatment with MNUA could be divided arbitrarily into three stages:

stage of cell depletion extending up to day 5; stage of regeneration up to day 30; and stage of neoplasia starting at day 40 after treatment. The microscopic changes correlated well with the changes in the thymus weight.

During the stage of cell depletion the cortex first showed pyknotic nuclei in thymic lymphocytes (Plates 1 and 2) and later marked hypocellularity resulting in an appearance sometimes described as thymic inversion (Plates 3 and 4). Some of the pyknotic nuclei of the cortical lymphocytes were phagocytized by the PAS positive reticular cells. The medulla and the epithelial and reticulum cells of the thymus did not show any detectable changes (Plate 4).

During the stage of regeneration the subcapsular zone of large lymphocytes was initially more prominent (Plates 5 and 6) and there was an increased mitotic activity in the cortex. The regeneration was complete histologically 30 days after treatment at which time no differences in the cellular composition of the cortex between control and experimental group could be seen. Some of the thymuses from the experimental group showed persistent increased mitotic activity in the cortex.

The features noted during the stage of neoplasia are summarized in Table I. The earliest neoplastic foci were seen at day 40. Three of the total of 44 thymic malignant lymphomas in the study were confined to the cortex of one lobule in one lobe of the thymus (Plate 7). Early

neoplastic changes in two adjacent lobules without disruption of the lobular septum was noted in two of these lobular tumours (Plate 8). Twenty-three tumours were confined to one lobe of the thymus. Five of these tumours involved only a part of the lobe and showed a background of thymic lymphocytes and intact medulla in one part of the lobe. Fifteen of the unilateral lobar thymic lymphomas showed an intact capsule (Plate 9) and a lobular pattern (Plate 10). Of the 18 bilateral thymic lymphomas six were small and were detected only on microscopic examination. Each lobe of the thymus in all these six animals showed an intact capsule in semiserial sections (Plate 11) and an absence of invasion from one lobe to the other. Also at day 100, two distinct and separate foci of neoplasia were seen in the two lobes of the thymus in one animal. It will be observed from Table I that from day 60 onwards 5 to 7 of the 10 animals examined at each time had a thymic malignant lymphoma. Compressed normal thymic tissue was not seen around any of the thymic lymphomas.

Twenty-seven of the thymic lymphomas showed the characteristic starry-sky pattern (Plate 12). PAS stain revealed that the large cells giving the starry-sky pattern were PAS positive. PAS positive cells were present in all tumours whether the starry-sky pattern was seen or not. An association of lymphoma cell mitoses with the PAS positive cells was apparent in screening the sections (Plates 13

and 14). A statistical analysis of this association could be done in 27 tumours. A statistically significant association between PAS positive cells and tumour cell mitoses ($p < .01$) was found in 20 tumours. The PAS positive cells also showed phagocytosis of pyknotic nuclei of tumour cells (Plate 14).

Imprints made from the thymic lymphomas showed that the predominant cell type in all but 2 of the malignant lymphomas was a large round cell (15 to 18 microns in diameter) with a thin rim of basophilic cytoplasm and a large reddish-purple nucleus showing clumping of chromatin and only occasional nucleoli. This predominant cell in the tumour imprint resembled the large lymphocyte or lymphoblast seen in the imprints of normal thymic cortex (Plates 15 and 16). The remaining two malignant lymphomas seen in the series were of the lymphocytic type.

Another feature noted during the stage of neoplasia was the formation of lymphoid follicles in the medulla (Table I and Plates 17 and 18). Lymphoid follicles were seen in the opposite lobe of the thymus in 11 of the 26 unilateral lymphomas. The lymphoid follicles were mature and consisted mostly of medium and small lymphocytes. In some instances they were quite large, impinging on the overlying cortex (Plate 18). There was almost complete replacement of medulla by tumour in the 18 bilateral thymic

lymphomas and therefore lymphoid follicles were not seen in these animals.

Spleen: Changes in the weights of the spleen after treatment with MNUA are shown in fig. 2. The spleen weight fell rapidly and significantly, but the recovery started at day 5 and was complete by day 10 (fig. 2). Because of the involvement of some spleens by malignant lymphoma the spleen weights showed marked scatter from day 100 after treatment.

Histologically the spleen at day 1 after treatment showed marked hypocellularity of the myeloid areas with moderate hypocellularity in the lymphoid follicles (Plates 19 and 20). These changes in the lymphoid follicles were less severe as compared with the thymus. The recovery of the myeloid and lymphoid areas was complete by day 10 and day 15 respectively. The earliest microscopic involvement of the spleen by malignant lymphoma was seen as irregular infiltration of both the myeloid and the lymphoid areas by tumour cells in 2 animals having grossly detectable thymic lymphomas (Plates 21 and 22). Early neoplasia and sequential changes indicative of the evolution of the neoplastic process comparable to those seen in the thymus were not observed at any time in the spleen. Involvement of spleen by malignant lymphoma was seen in 10 animals; all had grossly detectable thymic lymphomas.

Mesenteric lymph nodes showed minimal focal hypocellularity and lympholysis in the cortex at day 1 after treatment. Recovery appeared to have taken place by day 15. Changes suggestive of early neoplasia comparable to those seen in the thymus were not observed in the mesenteric lymph node. Involvement of mesenteric lymph node by malignant lymphoma was seen in 10 animals; all had grossly detectable thymic lymphomas.

Total and differential white cell counts: There was a rapid and significant fall in the total WBC and absolute neutrophil and lymphocyte counts 1 day after treatment (figs. 3 and 4). Recovery started between day 5 and 10 and the absolute neutrophil count returned to normal level by day 15 (fig. 3) while the absolute lymphocyte and total white cell counts returned to normal levels by day 40 after treatment (fig. 4). Thereafter the mean neutrophil and lymphocyte counts did not differ significantly from normal. Two mice with grossly detectable malignant lymphomas in the series showed leukaemia with total WBC counts of 19400 and 16800 and 70% and 30% blast cells. No mice with microscopic thymic lymphomas were leukaemic. Histology of the bone marrow in the two leukaemic mice showed marked involvement by the same neoplastic process as seen in the lymphoid organs (Plate 23). Of the 10 remaining mice with grossly detectable malignant lymphomas, 6 had 2 to 5% abnormal

cells in the peripheral smears, but their bone marrow was unremarkable histologically.

Extent of spread of malignant lymphoma: Besides the involvement of the thymus, spleen, mesenteric lymph node, and the bone marrow; the liver, the peripheral lymph nodes, the kidney and the lungs were also involved in eight of the twelve mice with full-fledged grossly detectable lymphomas. The liver showed infiltration of the portal areas while the peripheral lymph nodes were completely replaced by tumour. The lungs and the kidneys showed focal infiltration detectable only on microscopic examination. These organs were not examined in the mice having only microscopically detectable thymic lymphomas.

Unusual findings: In one instance malignant lymphoma was associated with chronic granulocytic leukaemia. The total white cell count was 16000 with 72% myeloid cells including myelocytes, metamyelocytes and neutrophils. The thymus in this animal showed lymphoma while the spleen and bone marrow showed diffuse proliferation of myeloid elements. In another mouse the spleen showed diffuse proliferation of predominantly erythroid elements with presence of normoblasts in the peripheral blood. The bone marrow of this mouse was unremarkable and the thymus did not show lymphoma.

Except for one malignant lymphoma involving the thymus spleen and lymph nodes seen in one of the control animals

sacrificed at day 150, the lymphoreticular and myelopoietic systems of the control group did not show any of the series of changes described above in the experimental group.

Autopsies on the animals that died during the course of the experiment and were thus not included in the sequential study revealed that all had malignant lymphomas with predominant involvement of the thymus and spread to the lymphoreticular and other organs. No control animals died before day 200.

B. Dose response relationships during development of malignant lymphoma induced by methylnitrosourea (MNUA)

1. Materials and Methods

Mice: Six to 7 week old female mice of the same strain as in the experiment on sequential morphological changes were used. They were kept under the same conditions as described for the previous experiment.

Carcinogen treatment: The preparation of solution of MNUA and method of injection were the same as for the experiment on sequential morphological changes. Four different single and 5 different fractional doses with 2 schedules were used. The single doses used were 25, 50, 75 and 100 mg per kg body weight (0.25, 0.50, 0.75 and 1 mmole/kg). The control group was given 0.4 ml of SSC

intraperitoneally. The fractional doses were 10, 20, 30, 40 and 50 mg per kg of body weight given daily for 5 days, or weekly for 5 weeks (total doses of 50, 100, 150, 200 and 250 mg or 0.50, 1, 1.5, 2 and 2.5 mmoles/kg respectively). The control groups received 0.4 ml of SSC per day or per week for 5 days or for 5 weeks respectively. About 30 mice were used in each of the control and experimental groups.

Parameters studied: The mice were observed for early mortality, i.e., death within 3 weeks of the last injection of MNUA and for incidence of malignant lymphomas with death as the end-point. The dead mice were autopsied and thymus, spleen and lymph nodes of mice which had malignant lymphoma were studied by routine histological methods. The experiment was terminated at 250 days after the first injection.

2. Results

1. Early mortality:

a) In single dose experiments early mortality was first seen at 75 mg/kg (Table II). In the fractionated dose group early mortality was first seen at the total dose of 200 mg/kg given as daily fractions and at the total dose of 250 mg/kg given as weekly fractions (Table III). A total dose of 100 mg/kg which produced 60% early mortality as a single dose did not produce any early mortality when given in daily or weekly fractions (Tables II and III).

b) It will be seen from fig. 5 that the 50% mortality point is at about 90 mg/kg for the single dose, at about 180 mg/kg for daily fractions and more than 250 mg/kg for the weekly fractions. The shift of 50% mortality point between the single dose and daily fractions is about 90 mg and more than 160 mg between single dose and weekly fractions.

2. Incidence of malignant lymphomas:

a) The highest incidence with the single dose group is 72% at the dose of 75 mg/kg (Table II) while that with daily doses is 82.7% at the total dose of 100 mg/kg and that with weekly doses is 93.3% at the total dose of 100 mg/kg (Table III).

b) Fractionation of 50 mg/kg dose reduced the incidence while fractionation of 100 mg/kg dose increased the incidence (Tables II and III). Weekly fractionation of all the doses except the total dose of 50 mg/kg gave consistently higher incidence than the daily fractionation (Table III).

c) It will be seen from fig. 5 that the 50% incidence point is at 40 mg for single dose and at about 60 mg for daily and weekly fractions. The shift of 50% incidence point between the single and the fractionated doses is about 20 mg.

d) In the single dose groups as well as the fractionated dose groups the incidence of lymphomas began to fall as early mortality appeared.

3. Latent period: In the single dose group latent period was shorter at 50 and 75 mg/kg doses than at 25 and 100 mg/kg doses (Table II). Fractionation of 50 mg dose resulted in prolongation while that of 100 mg dose resulted in shortening of the latent period (Tables II and III). At 150 and 200 mg/kg the weekly fractionated doses showed marked shortening of the latent period as compared with the daily fractionated doses (Table III).

4. Histology of malignant lymphoma: Because of post mortem autolytic changes, satisfactory histologic appraisal could be made in only 188 of the 205 lymphomas recorded in this experiment. Ninety-five per cent of these were lymphoblastic in type and the remaining 5% were lymphocytic. Predominant or exclusive thymic involvement was present in 93.7% of the tumours. Diffuse involvement of the spleen and of the lymph nodes and focal infiltrates of parenchymatous organs were also noted.

C. Serial transplantation of thymus cells during development of malignant lymphoma induced by 7,12-dimethylbenz [a] anthracene (DMBA)

1. Materials and Methods

Mice: Mice of the same strain and kept under the same conditions as described for the first two experiments were used.

Donor thymus cells were from animals injected within 12 hours of birth with 30 µg DMBA* dissolved in trioctanoin.* This dose was chosen because it gives a high yield of malignant lymphomas and has low early mortality (Ball and Dawson, 1969). The control donors received trioctanoin only. Recipients were ten day old normal animals of opposite sex to the donor of thymus cells. There is no sex-linked histoincompatibility in this strain of mice (Ball and Dawson, 1969).

Preparation and cell count of thymus suspension: The donor mice were killed by ether and their thymuses removed. A well dispersed suspension from each thymus separately was made by teasing with pointed forceps in Puck's saline A (sodium chloride: 8 g/l, potassium chloride: 0.4 g/l,

* Obtained from Eastman Organic Chemicals, Rochester, N.Y.

sodium bicarbonate: 0.35 g/l, glucose: 1 g/l of distilled water). The cells were washed once and resuspended in 0.5 ml of Puck's saline A; 0.1 ml of the suspension was diluted with appropriate amounts of 2% acetic acid for cell counts. The counts were made in the central area of a haemocytometer chamber until 2 counts with a difference of no more than 15% were obtained. Microscopic examination at the time of counting showed that the suspension consisted predominantly of single isolated cells with only occasional small clumps of 5 to 6 cells. From the time of removal to time of injection the cells were kept at ice-water temperature. This time interval was not longer than 1 hour.

Method of transplantation: Ten day old normal mice were anaesthetized with ether and were injected intravenously with thymus cell suspension of the donors of opposite sex. The injections were made via the right jugular vein by the method described by Griffith and Farris (1942). The suspension from one donor thymus was injected into one recipient mouse. Toe clipping was used for identification of individual mice. The mice were returned to the mother after recovery from anaesthesia and were weaned at 4 weeks.

Time intervals of transplantation: Donor thymuses from DMBA treated mice were transplanted as described

at 21, 28, 35, 42 and 60 days after treatment. These time intervals were chosen on basis of preliminary experiments (J. K. Ball, unpublished data). Thymuses from trioctanoin treated mice transplanted in a similar manner 28 and 60 days after treatment served as controls. Fifteen to 20 mice were used for both control and experimental groups at each point, i.e., thymuses from 15 to 20 mice treated with DMBA or trioctanoin were transplanted into each of 15 to 20 ten day old mice of opposite sex.

Transplantation of full-fledged thymic lymphomas:

Primary thymic lymphomas induced by 30 µg of DMBA given to new-borns were used. Total and differential WBC counts were done to determine the incidence of leukaemia in mice with lymphomas. The mice were then killed. Suspensions of thymic lymphomas were prepared and injected into 10 day old normal mice in the same manner as described above. Because of the possibility of presence of foci of necrosis in full-fledged thymic lymphomas, Trypan blue was used in cell counting, the exclusion of the dye being the criterion of viability. Suspensions containing 10^3 , 10^5 or 10^7 viable cells were injected. Suspensions containing required numbers of cells were prepared by serial dilution with Puck's saline A and in some cases normal thymus cells were added so as to make a total of 10^7 cells. A total of 9 primary thymic lymphomas were used-- 10^3 and 10^7 or 10^5 and

10^7 cells being transplanted from each tumour into 1 to 6 mice.

Parameters studied: The recipient mice were observed for the development of lymphomas. The following signs were looked for: holding of breath on holding by the neck, hunched position or palpable lymph nodes or spleen. A total and differential WBC count was made on mice with lymphomas or on moribund mice. The mice were then given 0.25 ml of 0.02% Colcemid* solution intraperitoneally. One and a half hours after Colcemid the mice were killed and a portion of the thymic lymphoma was used for karyotyping by the flame-drying technique (Bunker, 1965). It has been demonstrated previously in this laboratory that male and female somatic cells can be identified from the karyotypes in the CFW/D strain of mice (P. O. Ottonen, unpublished data). Similar observations have been made in other Swiss derived strains by Stich and Hsu (1960). The sex diagnosis by karyotyping depends upon the presence of three small chromosomes in the male cells and two in female cells. A complete autopsy was done to determine gross distribution of lymphomas. Imprints of thymic lymphomas and histology of thymus, spleen, lymph nodes, femoral bone marrow, liver and different parenchymatous organs were done to study the distribution

* Kindly supplied by Ciba Company Ltd., Pharmaceutical Division, Dorval, P.Q.

of lymphomas microscopically. The following terms were used to describe the distribution of tumour in the transplanted lymphomas: when there was marked gross enlargement of thymus with only slight gross or only microscopically detectable involvement of other organs the distribution of the lymphoma was described as predominantly thymic involvement. When the thymus was only microscopically involved, and other organs showed gross enlargement the distribution was described as moderate thymic involvement. When the thymus was the only organ of involvement by gross and microscopic examination the term exclusive involvement of thymus was used. The bone marrow involvement was detected by diffuse infiltration by lymphoma cells on histologic examination of several sections of femoral bone marrow. The term leukaemia was used when the differential count on the peripheral smear showed over 15% large abnormal blast cells with or without a high total WBC count.

Histology of thymus after DMBA treatment: Mice given 30 µg of DMBA within 12 hours of birth were killed by ether at 21, 28, 35, 42 or 60 days and the thymuses were removed for routine histological study to provide comparative information about microscopic changes in the thymuses transplanted at the same time intervals.

Termination of experiments: Recipient mice which did not develop malignant lymphoma were killed and autopsied at 250 days after transplantation.

2. Results

Histology of the thymus after treatment with DMBA:

The results are given in Table IV. It will be observed that consistent and progressive neoplastic changes in the thymus were seen from day 35 onwards after treatment with a final incidence of 75% at day 60. One mouse showed neoplastic changes at day 21. None of the mice had grossly detectable lymphomas until day 60 after treatment. The tumours were lymphoblastic in type and the extent of thymic involvement was the same as described in malignant lymphomas induced by single injections of methylnitrosourea (Joshi and Frei, 1970). Morphologically the cells of the neoplastic foci observed up to day 60 were indistinguishable from the cells of full-fledged thymic lymphomas. No foci of necrosis were observed in any of the neoplastic foci seen in this group. Neoplastic foci were not seen in the thymuses of trioctanoin treated mice.

Transplanted lymphomas: The incidence and other details of transplanted lymphomas occurring in the recipient mice after intravenous injection of thymus cells

at various times after DMBA treatment are given in Table V. Transplanted lymphomas were first observed in mice injected with donor thymus cells obtained at 35 days after treatment of donors with DMBA. The number of transplanted lymphomas increased to 44% (8 out of 18) when thymus cells of DMBA treated mice 60 days after treatment were transplanted. Karyotyping of these tumors showed that they were all of donor chromosome composition (Plates 24 and 25 and Appendix). The DMBA-induced primary thymic lymphomas were all transplantable when varying numbers of cells were injected except for one tumour which did not take after injection of 10^3 cells but took when 10^7 cells were injected. Karyotyping of 16 of 32 mice with transplanted lymphomas showed that the tumours were of donor chromosome composition.

The mice developing lymphomas after transplantation of thymus cells 35, 42 and 60 days after treatment showed exclusive or predominant involvement of the thymus and although 5 animals showed bone marrow involvement with leukaemia the remaining 14 showed no bone marrow involvement or leukaemia. All the mice developing lymphomas after transplantation of various numbers of cells of full-fledged thymic lymphomas on the other hand showed widespread involvement of lymphoreticular and parenchymatous organs and leukaemia. The peripheral counts varied from 8600 to 122000 with 18 to 84% blast cells.

Incidence of leukaemia in DMBA induced primary thymic lymphoma: Of the 22 mice with primary thymic lymphomas studied 4 showed leukaemia as well as involvement of bone marrow on microscopic examination giving an incidence of 18.1%.

Table I: Extent of intrathymic involvement and other features of thymic malignant lymphoma.

Day after* MNUA	Extent of involvement			Association of lymphoma mitoses with PAS positive cells p .01	Medullary follicles	
	Lobule	One Lobe	Both Lobes		with lymphoma	without lymphoma
40	1	1	0	2	0	1
60	1	3	0	4	2	7
80	1	4	0	5	1	2
100	0	6	1	7	3	0
120	0	3	2	5	2	0
140	0	1	6	7	4	3
160	0	3	3	6	5	1
180	0	1	4	5	2	0
200	0	1	2	3	1	0
Total	3	23	18	44	20	14

* Mice sampled before day 40 did not show neoplastic changes or medullary follicles.

Table II: Incidence of malignant lymphomas after a single dose of MNUA.

No.	Total Dose ^a	% Early Deaths ^b	Effective no. of mice ^c	% Lymphomas	Mean Latent Period (days)
1	0	0	30	0	-
2	25 mg	0	30	6.8	163
3	50 mg	0	24	58.3	144
4	75 mg	5	35	72	146
5	100 mg	60	12	33.3	174

^aDose given per kg body weight.

^bMice dying within three weeks after the carcinogen treatment.

^cEarly deaths excluded.

Table III: Incidence of malignant lymphomas after multiple doses of MNUA.

No.	Total Dose ^a	Schedule	% Early deaths ^b	Effective no. of mice ^c	% Lymphomas	Mean Latent Period(days)
1	0	0.4 ml SSC per day x5	0	30	0	-
2	0	0.4 ml SSC per week x5	0	30	0	-
3	50 mg	10 mg per day x5	0	30	20	185
4	50 mg	10 mg per week x5	0	29	6.9	218
5	100 mg	20 mg per day x5	0	28	82.7	139
6	100 mg	20 mg per week x5	0	30	93.3	147
7	150 mg	30 mg per day x5	0	28	71.4	146
8	150 mg	30 mg per week x5	0	28	89.3	97
9	200 mg	40 mg per day x5	86.6	4	50.0	179
10	200 mg	40 mg per week x5	0	29	93.1	87
11	250 mg	50 mg per day x5	93.3	2	0	-
12	250 mg	50 mg per week x5	30.8	18	83.3	90

^aDose given per kg body weight.

^bMice dying within three weeks after the last dose of the carcinogen treatment.

^cEarly deaths excluded.

Table IV: Extent of thymic involvement in primary DMBA lymphomas.

Treatment	Days after treatment	Lobular	Thymic Involvement		Total	% Incidence
			Lobar	Bilateral		
Trioctanoin	28	-	-	-	-	-
"	60	-	-	-	-	-
DMBA	21	0	1	-	1/10	10%
"	28	-	-	-	0/10	0
"	35	2 (20%)	2 (20%)	1 (10%)	5/10	50%
"	42	1 (12.5%)	3 (37.5%)	2 (25%)	6/8	75%
"	60	2 (16.6%)	5 (41.7%)	2 (16.6%)	9/12	75%

Table V: Characteristics of lymphomas occurring after transplatantation of thymus cells of DMBA treated mice.

	Thymus cells transplanted; days after treatment				Cells of full-fledged thymic lymphoma		
	21	28	35	42	60		
Average no.of cells	1.2x10 ⁷	0.5x10 ⁷	2.7x10 ⁷	2.6x10 ⁷	1.2x10 ⁷	1x10 ⁷	1x10 ⁵
Total no.of mice	19	17	19	18	18	14 ^a	9 ^b
No.of mice with trans-planted lymphoma	0	0	4	7	8	14	9
Karyotype	Donor				Donor		
Average latent period (days after trans-plantation)	-	-	57	65	56	10	24
Morphology	Lymphoblastic				Lymphoblastic		
Thymic involvement (no.of mice):							
Exclusive	-	-	2	3	4	0	0
Predominant	-	-	2	4	4	0	0
Moderate	-	-	0	0	0	14	9
WBC count (no.of mice):							
Leukaemic	-	-	2	1	2	14	9
Non-leukaemic	-	-	2	6	6	0	0

a = Nine thymic lymphomas transplanted using 1 to 2 mice for each.

b = Four thymic lymphomas transplanted using 2 to 3 mice for each.

c = Five thymic lymphomas transplanted using 1 to 6 mice for each.

d = Karyotype done on 16 of the 32 mice with transplanted lymphomas.

Fig. 1: Effect of MNUA on thymus weight.

X——X Mean thymus weights in mice given a single dose (75 mg/kg) of MNUA.

●——● Mean thymus weights in the control group; ten mice at each point.

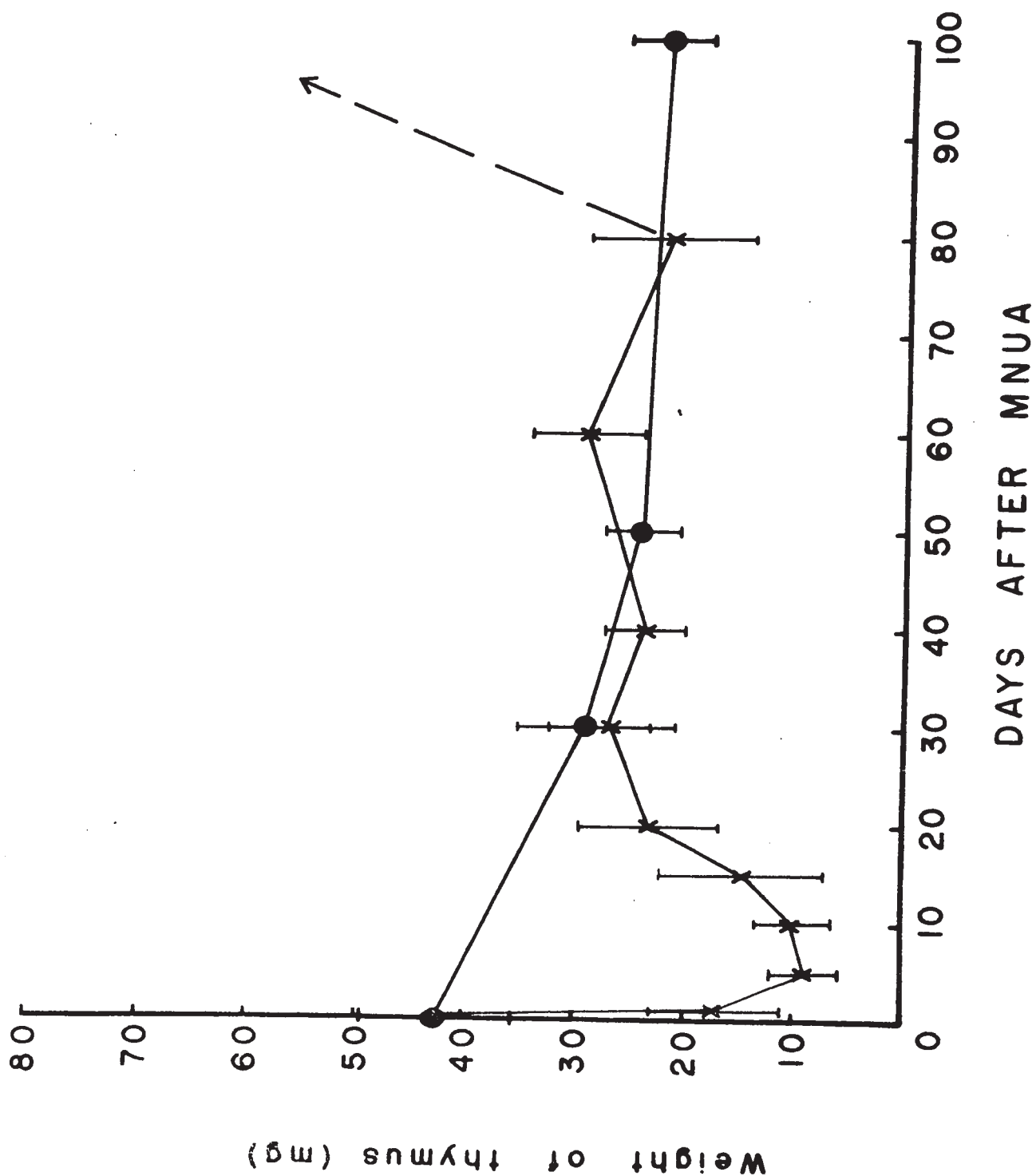


Fig. 2: Effect of MNUA on spleen weight.

X——X Mean spleen weight in mice given a
single dose (75 mg/kg) of MNUA.

●——● Mean spleen weight in the control
group. Ten mice at each point.

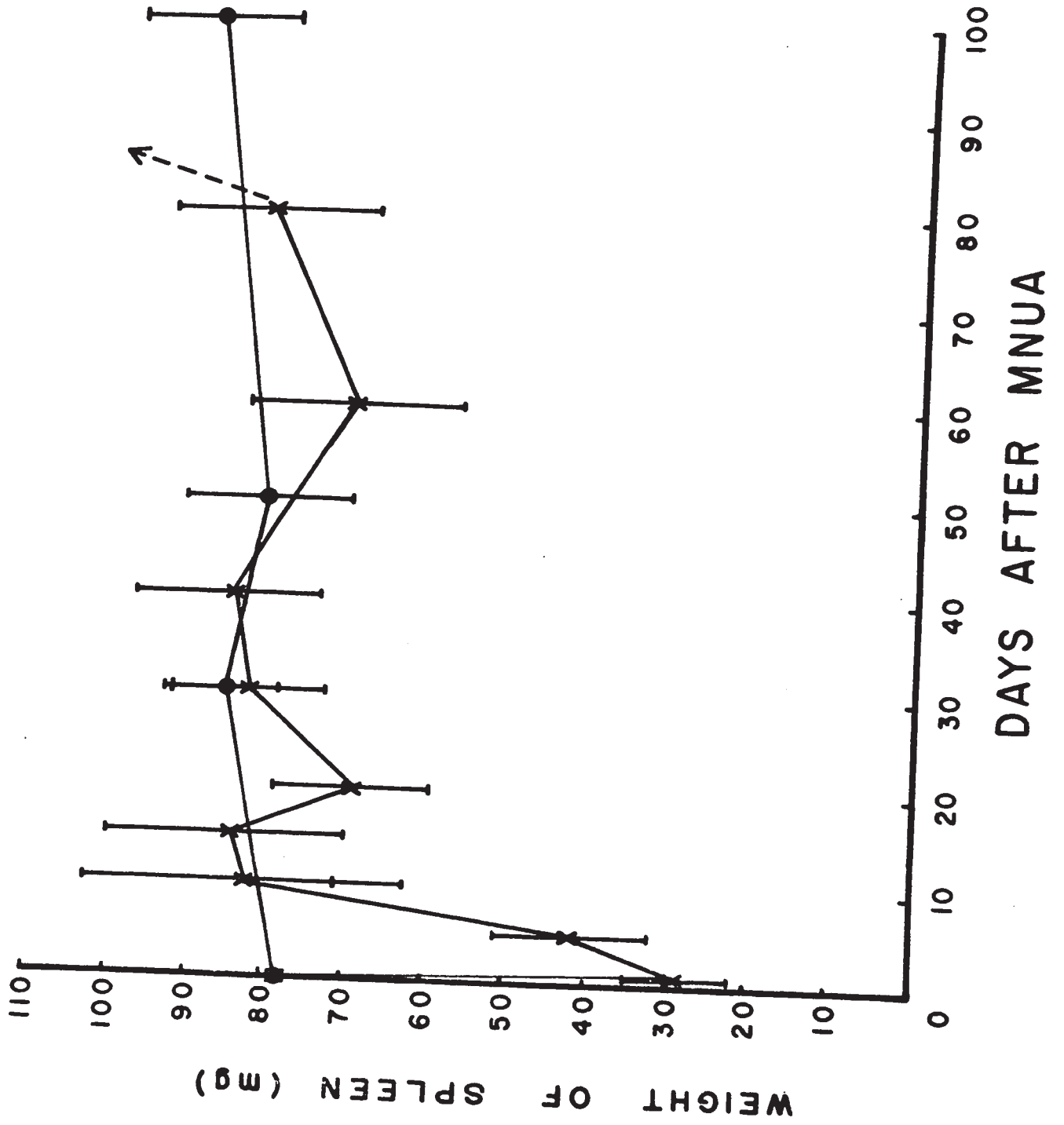


Fig. 3: Effect of MNUA on absolute neutrophil count.

●——● Mean absolute neutrophil count in mice given a single dose (75 mg/kg) of MNUA.

●-----● Mean absolute neutrophil count in the control group. Ten mice at each point.

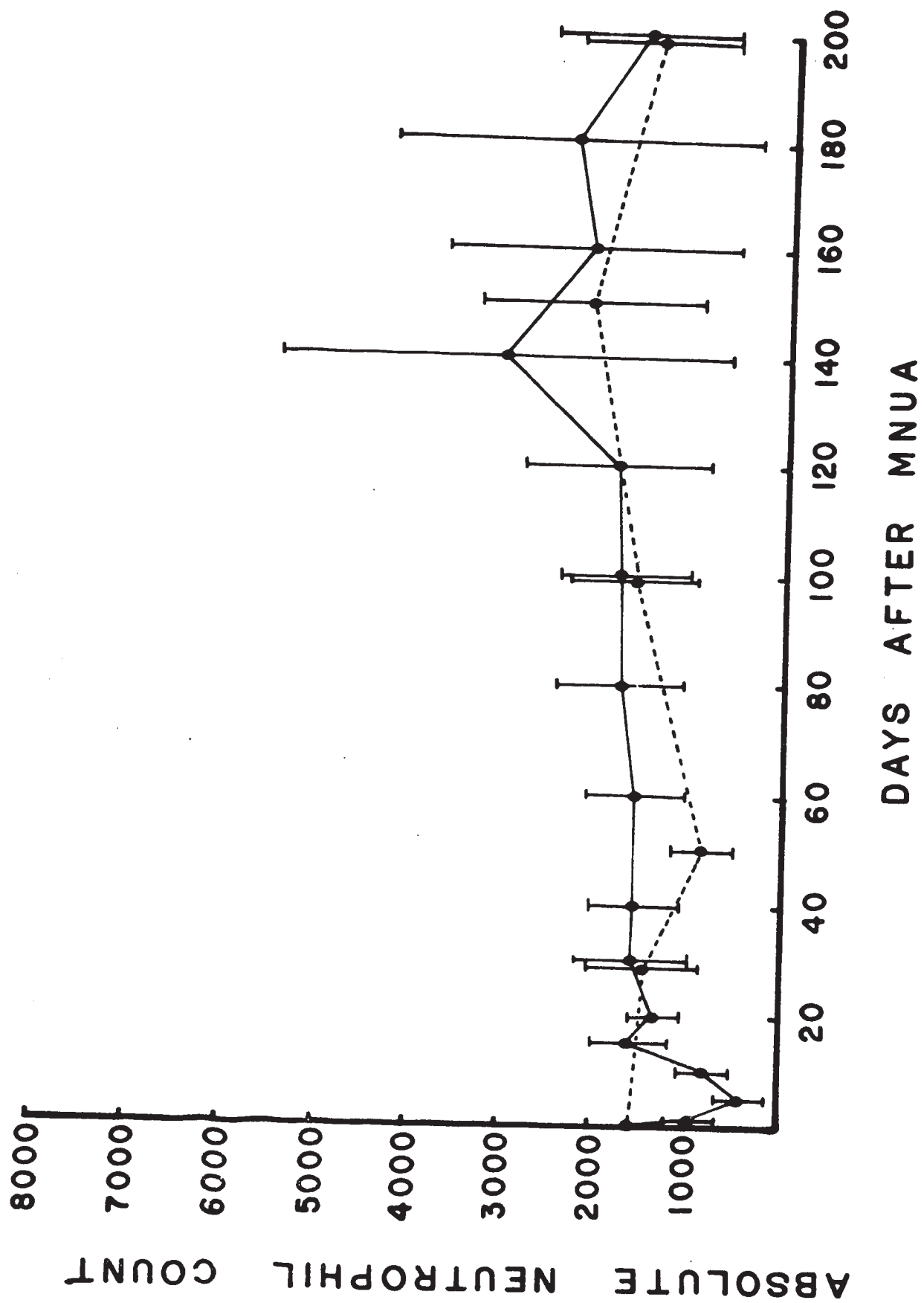


Fig. 4: Effect of MNUA on absolute lymphocyte count.

●——● Mean absolute lymphocyte count in mice given a single dose (75 mg/kg of MNUA.

●----● Mean absolute lymphocyte count in the control group. Ten mice at each point.

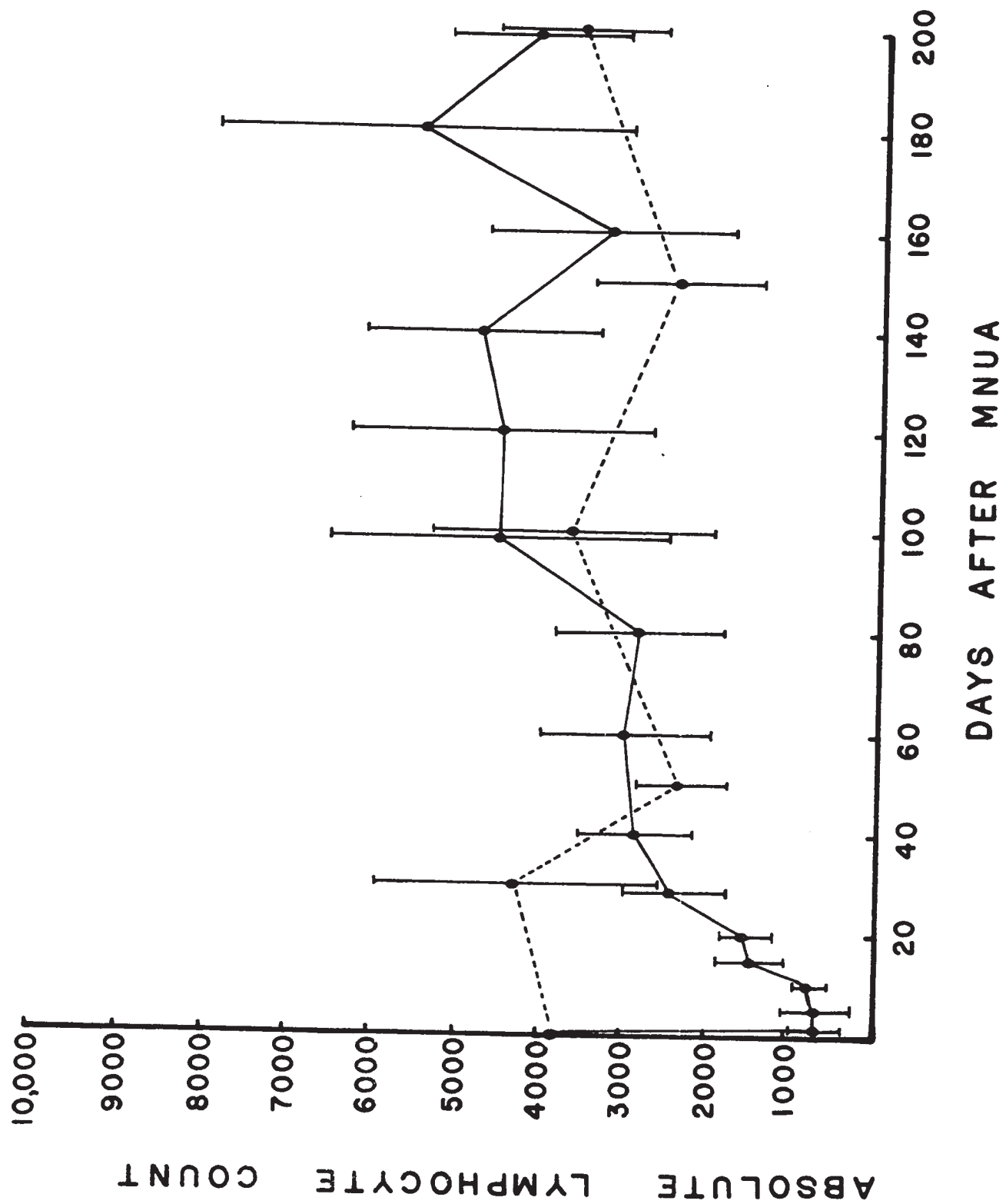
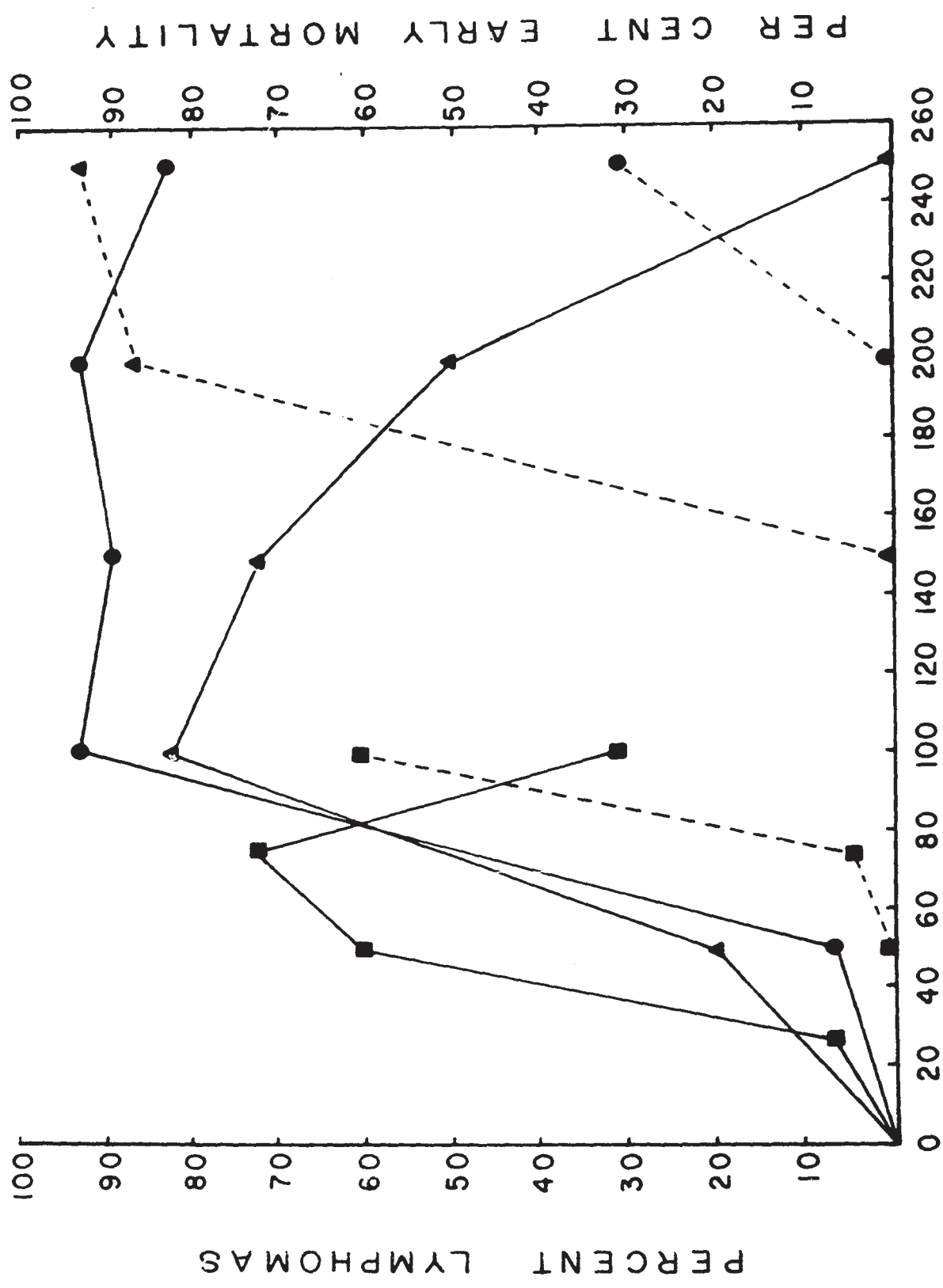


Fig. 5: Effect of single and fractionated doses of MNUA on the early mortality and incidence of malignant lymphomas.

Early mortality: ■-----■ single doses,
▲-----▲ total doses divided into five equal
fractions given at 1 day intervals, ●-----●
total doses divided into five equal fractions
given at 1 week intervals; incidence of
malignant lymphomas: ■————■ single doses,
▲————▲ total doses divided into five equal
fractions given at 1 day intervals, ●————●
total doses divided into five equal fractions
given at 1 week intervals.



TOTAL DOSE OF MNUA - mg / kg BODY WT.

Plate 1: Normal thymic cortex. H & E x 400.

Plate 2: Thymic cortex 1 day after treatment with MNUA. Note clusters of pyknotic nuclei (arrows). Upper right corner: a reticular cell phagocytizing pyknotic nucleus; cf. with figure 1. H & E x 400.



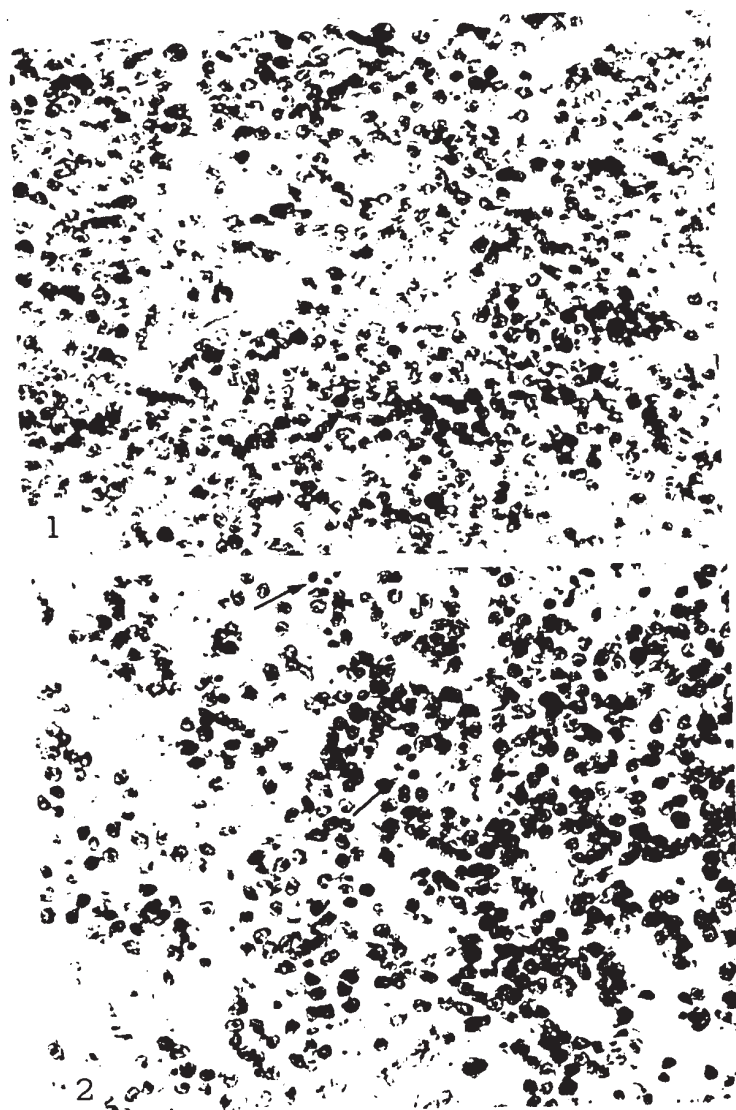


Plate 3: Normal thymus. H & E x 100.

Plate 4: Thymus 5 days after treatment with MNVA.
Note marked reduction in width and almost complete lymphocytic depletion of cortex with apparently unaffected medullary lymphocytes and reticular-epithelial cells; cf. with figure 3. This appearance is described as thymic inversion. H & E x 100.

3

4

3

4

Plate 5: Normal thymic cortex. H & E x 250.

Plate 6: Prominent subcapsular zone of large
lymphocytes 30 days after treatment with
MNUA; cf. with figure 5. H & E x 250.

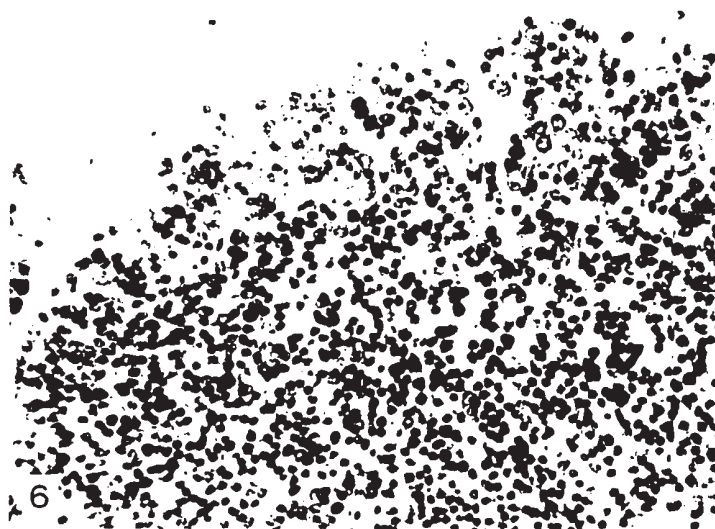


Plate 7: Early neoplastic focus in thymic cortex of MNUA-treated mouse. Note large uniform cells showing high mitotic activity; cf. size and appearance of cells with those in fig. 5. Lower left corner: intact medulla. There is a suggestion of early invasion of medulla. H & E x 250.

Plate 8: Lower half: lymphoma confined to one lobule. Far right of upper lobule: cluster of large uniform cells resembling cells of lymphoma. Rest of upper lobule (upper left) is normal. Lobular septum is intact. H & E x 400.



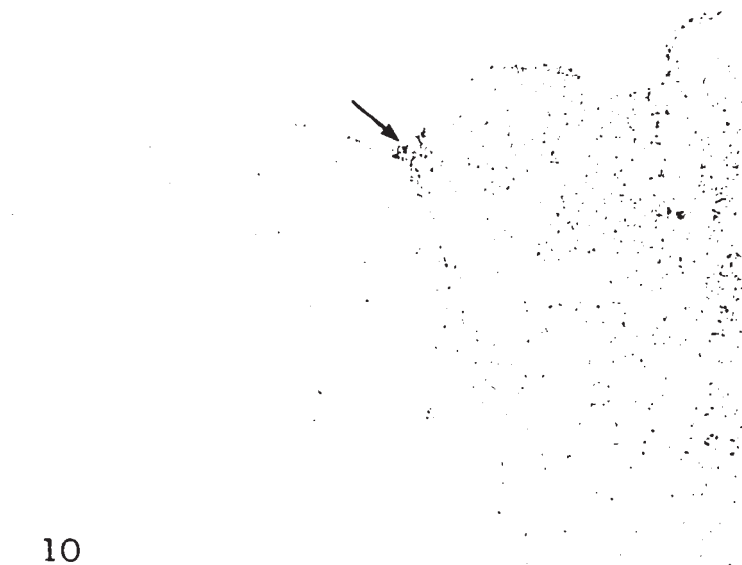
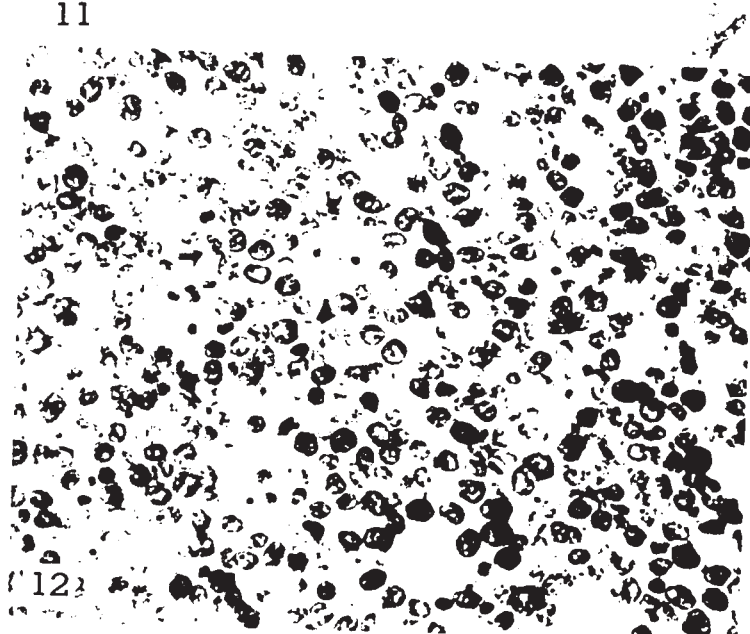


Plate 11: Lymphoma of both lobes of thymus. Note intact capsule of each lobe and persistent lobular pattern in one lobe. H & E x 40.

Plate 12: Starry-sky pattern of lymphoma. Many large pale cells show phagocytosis of nuclear debris. H & E x 400.

11



[12]

Plate 13: Large cell with rather indistinct notched nucleus (arrow). Two tumour cells close to this cell show mitosis. Periodic acid-Schiff. x 1000.

Plate 14: Large cell with vesicular nucleus (arrow). Tumour cells close to the large cell show mitosis. Phagocytosis by the large cell. Periodic acid-Schiff. x 1000.

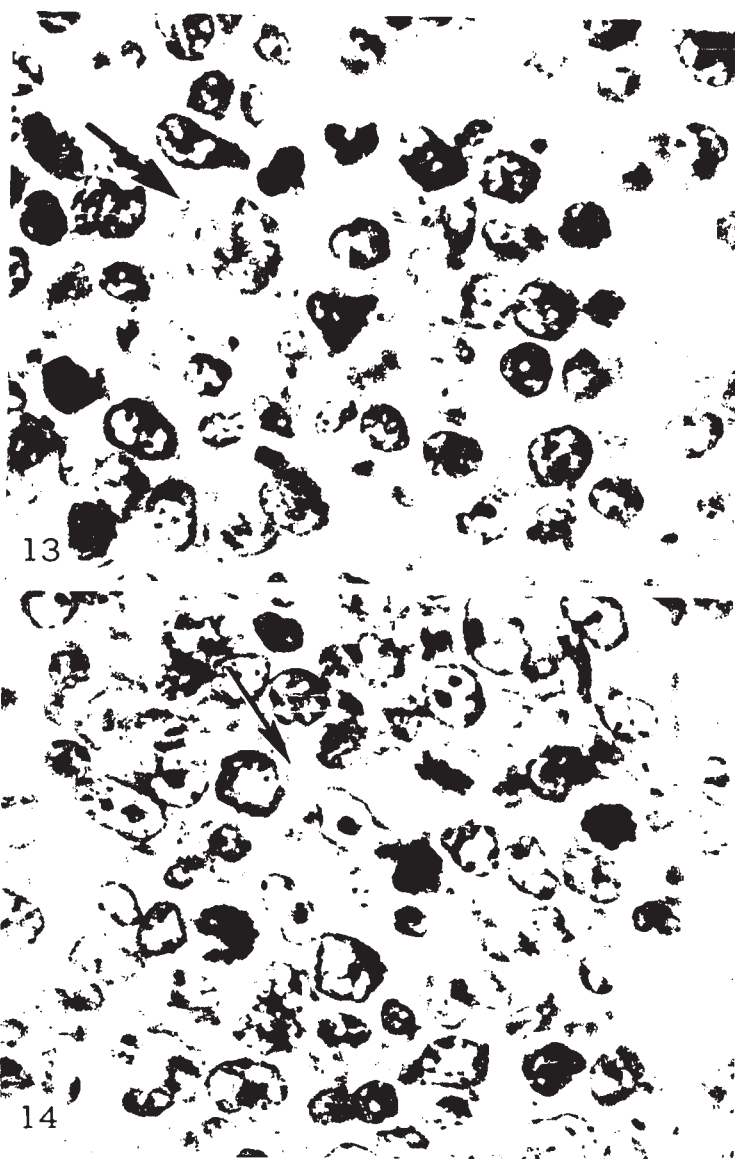


Plate 15: Imprint of superficial portion of normal thymic cortex. Numerous small and medium lymphocytes and a few large lymphocytes or lymphoblasts (arrow). Wright's. x 1000

Plate 16: Imprint of thymic lymphoma. Tumour cells resemble large lymphocytes or lymphoblasts in figure 15. A few small lymphocytes. Wright's. x 1000

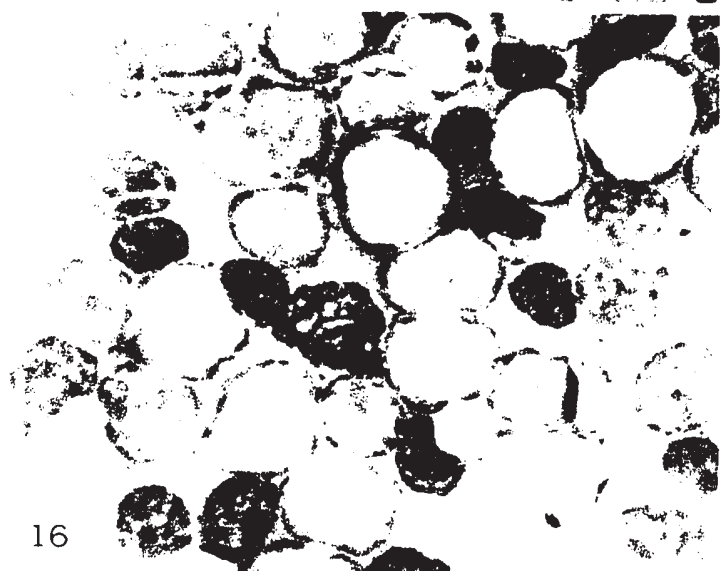
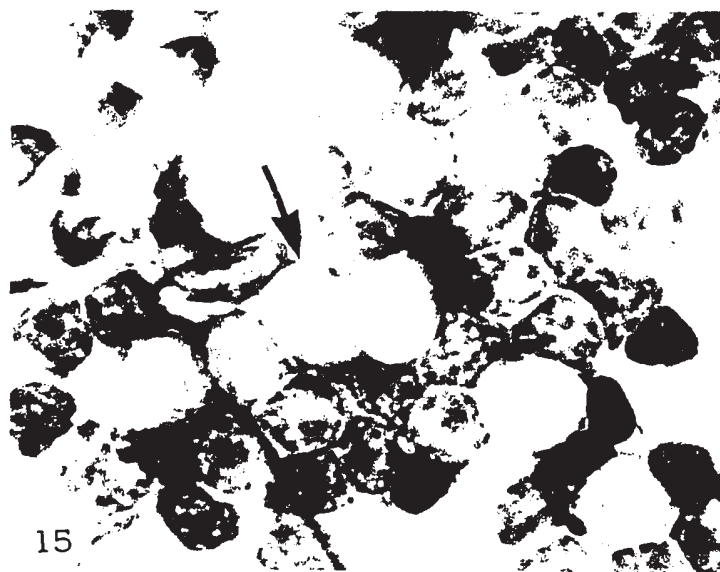


Plate 17: Normal thymus. Boundary between cortex and medulla is irregular. H & E x 100.

Plate 18: Large lymphoid follicle in medulla encroaching on cortex. Follicle is comprised of mostly small and medium lymphocytes; cf. with fig. 17.
H & E x 100.

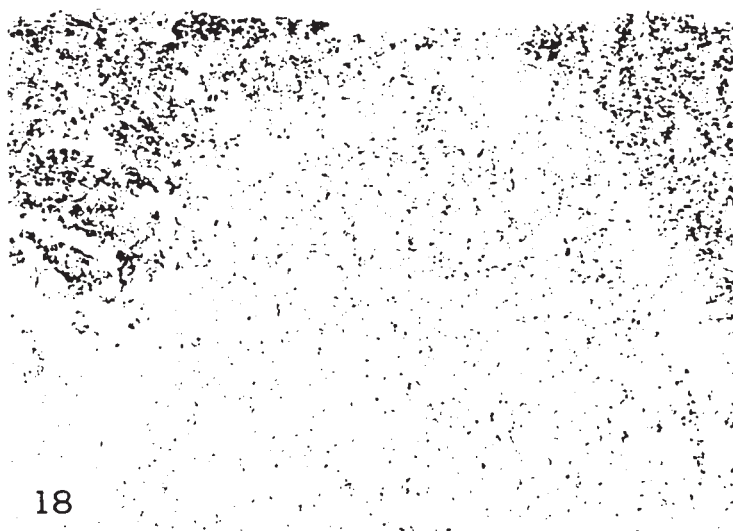
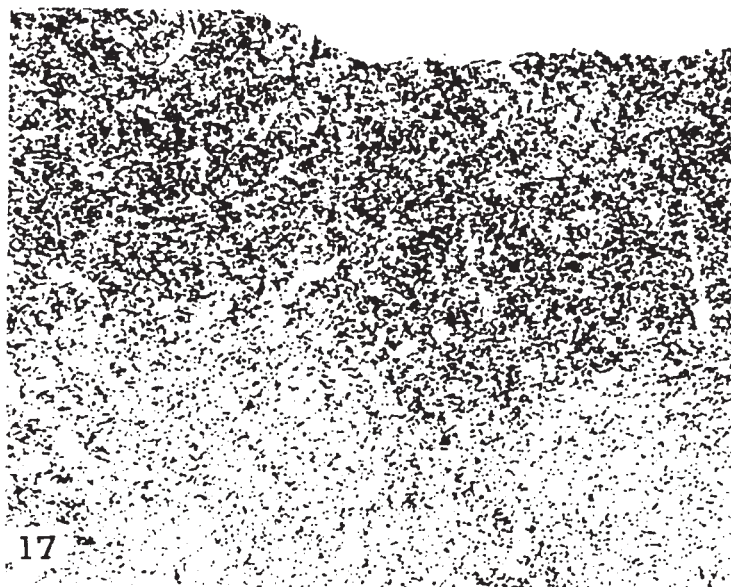


Plate 19: Normal spleen showing a lymphoid follicle and red pulp consisting of erythroid and myeloid elements. H & E x 100.

Plate 20: Spleen 1 day after treatment with MNUA. Note marked hypocellularity of red pulp. Lymphoid follicle also is hypocellular; cf. with fig. 19. H & E x 100.

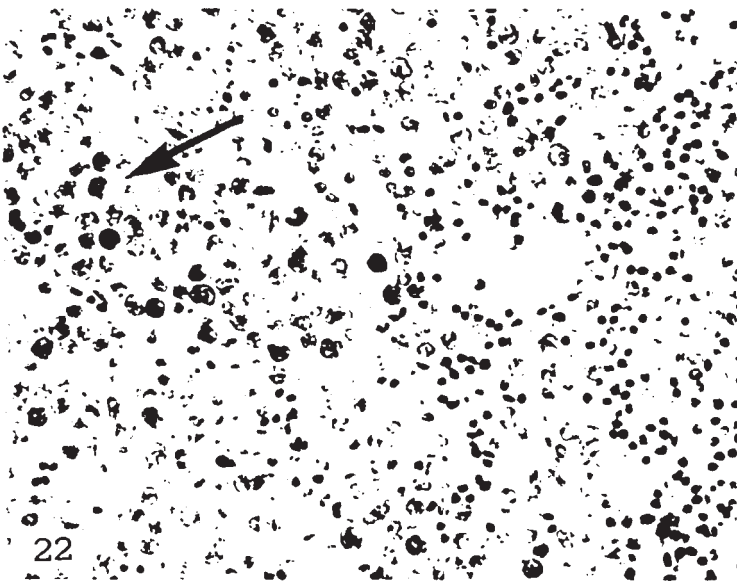
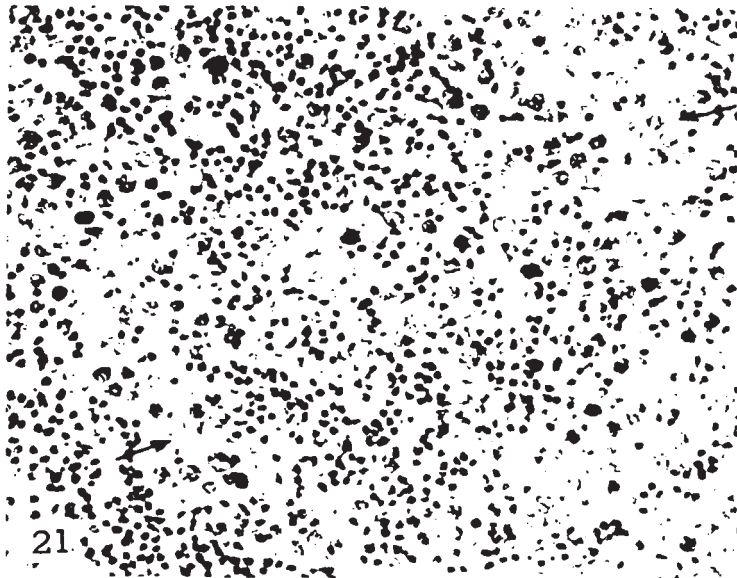


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Plate 21: Early involvement of lymphoid follicle of spleen by malignant lymphoma. Clusters of tumour cells (arrows) are infiltrating follicle. H & E x 250.

Plate 22: Early involvement of red pulp of spleen by malignant lymphoma. Clusters of tumour cells are infiltrating red pulp (arrow). Note groups of metamyelocytes. H & E x 250.



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Plate 23: Involvement of bone marrow by malignant lymphoma. Tumour cells have largely replaced normal elements of bone marrow.
H & E x 400.



Plate 24: Karyotype of thymic lymphoma in a female mouse occurring after transplantation of thymus cells of a male mouse 60 days after treatment with DMBA. There are 41 chromosomes. The arrows show three small chromosomes the smallest of which is probably the Y chromosome. The karyotype is characteristic of male. Giemsa stain x 1000.

Plate 25: Karyotype of thymic lymphoma in a male mouse occurring after transplantation of thymus cells of a female mouse 35 days after treatment with DMBA. There are 41 chromosomes. The arrows show two small chromosomes. The karyotype is characteristic of female. Giemsa stain x 1000.



IV. DISCUSSION

The sequential gross and microscopic changes in the lymphoreticular system during the development of malignant lymphoma induced by a single injection of MNUA suggest that the probable sequence of events is as follows: Following the treatment with MNUA there is initially severe cell depletion in the thymic cortex, splenic red pulp and bone marrow as indicated by rapid fall in the white cell counts and weights (Figs. 1 to 4). The cell depletion seen in the lymphoid follicles of the spleen and in the mesenteric lymph node is comparatively less severe. All these changes are similar to the effects of DMBA in mice (Rappaport and Baroni, 1962; Ball and Dawson, 1969). The changes in the thymus are similar to those seen in the initial stages of genesis of virus and radiation-induced malignant lymphomas in mice (Dunn, Moloney, Green and Arnold, 1961; Wasi and Block, 1961). In AKR mice Siegler and Rich (1963) observed only unilateral cell depletion in the thymus and Metcalf (1966) has reported occurrence of medullary enlargement prior to cell depletion in the thymic cortex.

The stage of cell depletion in the lymphoreticular system in the genesis of MNUA-induced malignant lymphoma is followed by the stage of regeneration with return to normal as judged by organ weights, histology and cell counts (Figs. 1 to 4). The stage of regeneration is similar to that observed after DMBA treatment in new-born mice (Rappaport and Baroni, 1961). The regeneration after MNUA treatment of adult mice however is apparently complete while Ball and Dawson (1969) have reported that in DMBA treated new-born mice thymus weights and bone marrow cell counts fall short of complete recovery and that DMBA-treated bone marrow fails to restore immunosuppression produced by DMBA treatment. The stage of regeneration occurs in Gross virus-induced and in radiation-induced lymphomas (Goodman and Block, 1963; Wasi and Block, 1961; Siegler, Harrell and Rich, 1966), but not in the lymphomas of AKR mice (Siegler and Rich, 1963; Metcalf, 1966). In the AKR mice the stage of cell depletion in the thymus is followed by the stage of neoplasia.

The stage of regeneration is followed by the stage of neoplasia in the MNUA-induced malignant lymphoma. As in virus (Arnesen, 1958; Dunn et al., 1961; Goodman and Block, 1963; Siegler and Rich, 1963; Metcalf, 1966), DMBA (Rappaport and Baroni, 1962), and radiation-induced lymphomas (Carnes and Kaplan, 1956; Wasi and Block, 1961; Siegler et al., 1966), the thymus is the primary site of the malignant lymphoma.

The initial neoplastic changes were seen in the cortex. In virus (Dunn et al., 1961; Metcalf, 1966), radiation (Carnes and Kaplan, 1956; Wasi and Block, 1961), and DMBA-induced malignant lymphomas (Rappaport and Baroni, 1962), a similar observation has been made. Study of the evolution of neoplastic changes in the thymus in MNUA-induced malignant lymphoma reveals that separate foci of neoplasia in adjacent lobules or opposite lobes of the thymus occur at least in some instances without the disruption of lobular septa (Plate 8) or capsules of the lobes of the thymus (Plate 11). The persistence of medulla and lobular pattern in the early unilateral thymic lymphomas (Plate 10), the presence of separate foci of neoplasia in the two lobes, the absence of actual invasion of a lobe by a neoplastic focus in the opposite lobe and the intact capsules of each lobe in early bilateral thymic lymphomas were also noted (Plate 11). These observations suggest that at least in some instances the lymphoma in the thymus arises as more than one focus and that these later become confluent. This conclusion is also supported by the fact that the MNUA-induced thymic lymphoma in most instances retained the shape of the normal thymus and that compressed remnants of thymic tissue were not seen in any of the thymic lymphomas. Levinthal, Buffett and Furth (1961) also detected multiple foci of neoplasia becoming confluent and "transforming the whole

thymus into lymphomatous tumour." This observation is in contrast to the unilateral origin of thymic lymphoma followed by invasion of the opposite lobe as is described in AKR mice and radiation-induced lymphoma (Siegler and Rich, 1963; Siegler et al., 1966).

Study of dose-response relationship in the development of MNUA-induced malignant lymphoma shows that reduced mortality, enhancement of tumour incidence and shortening of latent period are the main effects of fractionation of doses of MNUA (Fig. 5 and Table III). Similar observations have been made in radiation-induced malignant lymphoma in mice (Kaplan and Brown, 1952), in DMBA-induced mammary tumours in rats (Huggins and Grand, 1968; Shimkin, Gruenstein, Meranze and Thatcher, 1969), and in DMBA-induced skin tumours in mice (Salaman and Roe, 1956; Vesselinovitch, 1958). Kaplan and Brown (1952) found that the pattern of fractionation also influenced the incidence of tumours; 4 to 8 day intervals gave the maximum yield of radiation-induced malignant lymphomas. Shimkin et al. (1969) found that weekly doses were more effective in enhancement of DMBA-induced mammary tumours of rats than daily doses. In the present study, the optimum dose for getting a high tumour yield in the shortest possible time was 150 mg given as five equal weekly fractions.

The precise mechanisms of the enhancement of tumour incidence and shortening of latent period are not known.

Kaplan and Brown (1952) suggested that in radiation-induced malignant lymphomas the increased incidence could be related to the timing of each successive dose with respect to the rhythm of cell depletion and regeneration in the thymus induced by each preceding dose. Wasi and Block (1961) and Siegler et al. (1966) have shown that cell depletion in the thymus after each dose of radiation is highest at 48 hours and regeneration begins after that period. For MNUA it has been previously shown (Frei, 1970; Joshi and Frei, 1970) that cell depletion in the thymus is maximal at 3 to 5 days and the regeneration begins after that period. Thus there may be a correlation between the rhythm of cell depletion-regeneration and the high incidence of tumours obtained in the present experiment after administration of weekly fractionated doses. However, high incidence of lymphomas was also obtained by daily fractionated doses at 100 and 150 mg and this explanation does not hold good for these dosage levels. The higher incidence of malignant lymphomas noted in the fractionated doses may also be indirectly related to reduced mortality, perhaps by a lack of killing of the "target" cells which ultimately undergo neoplastic change.

A marked shortening of the latent period was observed with weekly fractionated doses (Table III). This effect may be due to higher rate of growth of the participating

clones of tumour cells or to recruiting of new clones of tumour cells during the later stages of carcinogenesis as suggested by Blum (1950; 1959a; 1959b) in describing the mechanism of cancer induction by successive doses of ultra-violet radiation. Another factor responsible for reduction of latent period may be suppression of immune response. Friedrich-Freska and Hoffman (1969) have shown that the latent period of dimethylnitrosamine-induced hepatic tumours in rats is reduced by continuous administration of anti-lymphocytic serum (ALS). They observed that one of the factors related to the latent period of these hepatic tumours of rats may be an immunological reaction on the part of the host against the emerging neoplastic cells and the reduction in the latent period seen after ALS treatment may be due to suppression of the reaction. Chemical carcinogens such as DMBA, methylcholanthrene, benz a pyrene (Ball, 1970) and MNUA (J. K. Ball: personal communication) are known to have immunosuppressive action. It is possible that by giving weekly fractionated doses of MNUA[a] sustained suppression of the immunological reaction is obtained resulting in a reduction of the latent period.

Only a small shift of the 50% tumour incidence point as compared with the marked shift of the 50% mortality point (Fig. 5) suggests that the changes in the lymphoid tissue leading to neoplasia are more persistent.

If the different phenomena discussed above are indeed related to the increased incidence and shortening of the latent period of malignant lymphomas seen after fractionation of doses of MNUA they would support the "multiple-hit" or "multiple-stage" theory of carcinogenesis which postulates that multiple changes are necessary for the manifestation of a neoplastic potentiality (Ashley, 1969). Thus the rhythm of cell-depletion and regeneration, the acceleration of the growth rate of the participating clones of tumour cells, the recruitment of new clones of tumour cells, a possible "feeder effect" of PAS positive reticular cells of the thymic cortex (Joshi and Frei, 1970), an immunological defence reaction, and a possible activation of virus may all be the factors involved in the genesis of malignant lymphoma induced by fractionated doses of MNUA.

Serial transplantation of thymus cells during the genesis of DMBA-induced malignant lymphoma has shown that some of them are transplantable from 35 days onwards after treatment (Table V). Histological evidence of neoplastic changes was also seen consistently from 35 days onwards after treatment (Table IV). Previous serial transplantation studies on viral-induced lymphomas by Levinthal et al. (1961), Haran-Ghera, Liberman and Kaplan (1966) and Metcalf (1966) have also shown correlation between the first appearance of histological changes and transplantability.

Levinthal et al. (1961) found that definite histologic evidence of neoplasia was seen 50 days after injection of Gross virus in AK/Z mice and the definite positive tumour bioassays by intramuscular grafting were seen from 57 days onwards after treatment. Haran-Ghera et al. (1966) reported that intra-renal thymus grafts when directly inoculated with RadLV showed histologic evidence and positive tumour bioassay as early as one week after inoculation of virus. Metcalf (1966) found that the thymuses from AKR mice showing histologic evidence of early neoplasia grow progressively as tumours when transplanted intramuscularly.

The extent of correlation varies depending upon the system used and the time after treatment at which tumour bioassay is done. Thus Levinthal et al. (1961) reported a correlation of "somewhat less than 100%," Metcalf (1966) about 87% and Haran-Ghera et al. (1966) from 33 to 100% at various times after treatment. In the present study the extent of correlation at day 60 after treatment is about 55% (75% of the total number of animals examined showed histologic evidence of neoplasia; 44% of the total number of animals receiving 60 day old DMBA treated thymus cells showed transplanted lymphoma). The reasons for the correlation being lower than in other studies referred to above are not clear. However, the following factors may be responsible. At 60 days after DMBA treatment about 16%

of the animals still showed lymphomas confined to one lobule of the thymus (Table IV). These tumours may be too small to be transplantable when injected intravenously. This also suggests that at day 60 the neoplastic changes were still in their early stages of development and this time interval may not be comparable with the time intervals of studies reported by Levinthal et al. (1961), Haran-Ghera et al. (1966) and Metcalf (1966).

The distribution of the transplanted tumours occurring after thymus cells at various times after DMBA treatment could be indicative of the site or sites where the thymus cells lodge or attach and proliferate. The thymuses of DMBA-treated mice showed only microscopically detectable lymphoma until 60 days after treatment. The average latent period of lymphomas induced by a single injection of 30 µg of DMBA is about 110 days (Ball and Dawson, 1969). The neoplastic cells of the thymuses of DMBA-treated mice 35, 42 and 60 days after treatment could therefore be referred to as early neoplastic cells. Cells of the thymuses of mice with lymphomas showing gross enlargement of the thymus producing breathlessness and imminent death could be referred to as full-fledged neoplastic cells.

Although there is some overlap, the results of serial transplantation of thymus cells suggest that the early neoplastic cells attach and proliferate exclusively or predominately in the thymus. This is shown by exclusive

or predominant involvement of the thymus in lymphomas occurring after transplantation of such cells (Table V). On the other hand the full-fledged neoplastic cells when injected in varying numbers attach and proliferate in the bone marrow and thymus as shown by the leukaemic blood picture and histological demonstration of bone marrow and thymus involvement (Table V). Ioachim, Cali and Sinha (1965) injected intraperitoneally leukaemic lymphoblasts harvested from tissue cultures of Gross virus induced thymic lymphoma in rats. The lymphoma occurring in the recipient rats localized mainly in the parathymic lymph nodes with secondary involvement of the thymus. The authors suggested that this localization may be due to affinity of leukaemic lymphoblasts to colonize and proliferate in this area. The capacity of differential attachment and proliferation of bone marrow and lymphoid cells has been referred as "homing" capacity by Ford (1966). The normal thymus cells "home" to the lymph nodes and spleen and not to the thymus or bone marrow (Ford, 1966; Micklem and Loutit, 1966). Thus there is a suggestion of differences in the homing capacity of early and full-fledged neoplastic cells as compared with the normal thymus cells. The evidence presented is however not conclusive because the differences in the homing capacity as indicated by distribution of transplanted lymphomas may be related to different numbers of early and late neoplastic cells transplanted.

This point could be clarified by carrying out further experiments in transplantation of various numbers of full-fledged neoplastic cells mixed with various numbers of normal thymus cells.

Early and progressive neoplastic changes comparable to those seen in the thymus were not seen in the spleen and in the mesenteric lymph node after treatment with MNUA. Neoplastic involvement of the spleen and mesenteric-lymph node was seen only in those mice which had gross thymic malignant lymphoma. There is evidence that mouse thymus cells migrate to the lymph nodes and perhaps to the spleen (Miller, 1963; Ford, 1966; Micklem and Loutit, 1966). It is possible that the spread of malignant thymic lymphoma cells to the lymph nodes and the spleen may be related to the traffic of lymphoid cells out of the thymus. All these observations suggest that the involvement of the spleen and the lymph nodes is by the process of blood-borne metastasis from the thymic malignant thymoma rather than by separate foci of neoplasia. Rappaport and Baroni (1962) have expressed the same view in the DMBA-induced malignant lymphomas. In carcinomas and sarcomas also, spread of the disease occurs by the process of blood-borne metastasis. The main differences in the distribution of metastatic lesions in the lymphoid and non-lymphoid tumours are worth noting. In the lymphoid tumours the spleen is mainly and almost consistently involved while the lungs show only focal

involvement often detectable only on microscopic examination. In the non-lymphoid malignant tumours (carcinomas and sarcomas) on the other hand lungs are mainly and almost consistently involved while the spleen is an uncommon site of metastasis. These differences can be explained by postulating that in lymphoid tumours the process of metastasis is probably related to the normal traffic and "homing" capacity of lymphoid cells and the tumour cells are released into the blood stream as single cells or as extremely small clumps which easily pass through the pulmonary circulation. As discussed above, transplantation studies of full-fledged neoplastic cells of DMBA-induced thymic lymphoma suggest that full-fledged neoplastic cells "attach and proliferate" or "home" to various lymphoreticular tissues (Table V). It is possible that the cells of MNUA-induced malignant lymphoma have similar characteristics which would explain the spread of malignant lymphoma to various tissues of the lymphoreticular system.

The difference in the distribution of lymphomas occurring after transplantation of early and full-fledged neoplastic thymus cells (Table V) is compatible with the phenomenon of tumour progression as described by Foulds (1954). It appears that early neoplastic cells proliferate only in the thymus whereas full-fledged neoplastic cells are autonomous and proliferate in the bone marrow and various lymphoid organs. About 18% of the animals having DMBA-induced

lymphoma showed leukaemia. Also two instances of leukaemia were seen in association with MNUA-induced malignant lymphoma. The occurrence of this leukaemic phase or leukaemic transformation of malignant lymphoma may be related to the progression with respect to the homing capacity and autonomy of neoplastic thymus cells. It would appear that in DMBA-induced and also perhaps in MNUA-induced malignant lymphomas leukaemia supervenes when the lymphoma cells become autonomous and acquire the capacity of homing to the bone marrow.

The elucidation of the role of bone marrow in the initial stages of MNUA-induced lymphoma is beyond the scope of this study; however, in view of the constant influx of bone marrow cells to the thymus that probably occurs normally in the mouse (Ford, 1966) and the findings of Ball (1968) in DMBA-induced malignant lymphoma and of Lorenz, Law and Congdon (1954) in AKR mice, the bone marrow may play a role in the initial stages of MNUA-induced malignant lymphoma.

It has not been sufficiently emphasized in most reports on the pathogenesis of neoplastic lesions of the lymphoreticular system that although an individual organ or tissue such as the thymus as in malignant lymphomas or the spleen in erythroleukaemia (Metcalf, Furth and Buffett, 1959; Rauscher, 1962), or the lymph nodes in reticulum cell sarcoma (Siegler and Rich, 1968), is the site of origin of

these different neoplastic processes, all the organs and tissues of the lymphoreticular system are affected during the genesis of most of these tumours (Dunn et al., 1961; Wasi and Block, 1961; Rappaport and Baroni, 1962; Ball and Dawson, 1969). In view of the normal traffic of cells from and to the different organs of the lymphoreticular system that probably occurs normally in mice (Ford, 1966; Micklem and Loutit, 1966), the lymphoreticular system may act as a closely integrated system in the genesis of the neoplastic process produced by different agents. The unusual manifestations of association of MNUA-induced malignant lymphoma and chronic granulocytic leukaemia in one instance and occurrence of diffuse proliferation of predominantly erythroid elements in the spleen in another instance may be illustrations of the interrelationship between the disorders of different tissues of lymphoreticular system and may support the above contention.

The starry sky pattern (Plate 12), the association of mitoses in the tumour cells with the PAS positive cells in MNUA-induced malignant lymphomas (Plates 13 and 14) and the occurrence of medullary lymphoid follicles during their development (Plate 18) may have some immunological significance. The starry sky pattern and the large histiocytes are also seen in Burkitt's lymphoma, cat and dog lymphomas and lymphomas in AKR mice (Metcalf, Ishidate and Brumby, 1967). There is some circumstantial evidence to suggest that an

antigenic factor may be involved. Thus lymphocyte cultures from patients of rheumatoid arthritis (Bartfeld and Juliar, 1964), and systemic lupus erythematosus (Marmont and Damasio, 1965) show rosettes consisting of a large central macrophage surrounded by lymphoid cells, an appearance reminiscent of starry sky pattern. Similar pattern is seen in explants of lymph nodes and spleen obtained from rabbits subjected to antigenic stimulation (Sharp and Burwell, 1960). In the cultures the lymphocytes show DNA synthesis while the tumour cells around the PAS positive cells in the malignant lymphoma show mitosis. Though the exact functional relationship between the PAS positive cells and the tumour cells is not clear, the resemblance of the pattern to the one seen in cultures and explants of lymphoid tissues in immunological disorders suggests that the association may be related to immunological phenomena. The exact origin and nature of these PAS positive cells could not be ascertained but as Metcalf et al. (1967) have suggested it is possible that they might be derived from the local reticular cells. Lymphoid follicles in the thymic medulla are also observed in such immunological disorders as autoimmune haemolytic anaemia in NZB mice (Burnet and Holmes, 1964), and in myasthenia gravis in man (Castleman, 1966). In the genesis of MNUA-induced malignant lymphoma follicles in the thymic medulla may be a local immune response to a tumour antigen or perhaps to a virus.

In the MNUA-induced malignant lymphomas there are many features which are also seen in virus-induced malignant lymphomas, e.g., occurrence of cell depletion as in Gross virus-induced lymphoma in C3H mice (Goodman and Block, 1963) and in AKR mice (Siegler and Rich, 1963; Metcalf, 1966), regeneration in Gross virus-induced lymphoma in C3H mice (Goodman and Block, 1963), occurrence of association of PAS-positive cells with mitosis in tumour cells and the formation of lymphoid follicles in the medulla as in malignant lymphoma in AKR mice (Metcalf, 1966). These similarities suggest that a virus may be involved in the genesis of MNUA-induced malignant lymphoma as also probably occurs in other chemically-induced malignant lymphomas (Irino et al., 1963; Toth, 1963; Ribachi and Giraldo, 1966; Haran-Ghera, 1967). For this reason, in further studies attempts should be made to transfer the disease by cell-free filtrates to other strains of mice and at fine structure examination for the presence of virus particles.

In this study the sequential gross and microscopic changes in the lymphoreticular system occurring during the genesis of MNUA-induced malignant lymphoma have been described for the first time and it is shown that all the tissues of the lymphoreticular system are affected during the genesis of lymphoma. The dose response relationships during the genesis of MNUA-induced malignant lymphoma were studied for the first time and it was shown that reduced

early mortality, enhanced tumour incidence and shortening of the latent period are the main effects of fractionation. Serial transplantation of thymus cells during the genesis of DMBA induced malignant lymphoma was carried out for the first time and it was shown that the thymus cells first become transplantable 35 days after treatment with DMBA. The cells of origin of the transplanted tumour was demonstrated by using karyological methods for the first time. A detailed study of the distribution of the transplanted lymphomas was done for the first time and it was found that the majority of the malignant lymphomas occurring after transplantation of early neoplastic cells involve exclusively or predominantly the thymus and those occurring after transplantation of full-fledged neoplastic cells involve both the bone marrow and the thymus.

In further studies attempt should be made to transfer the disease by cell-free filtrates of MNUA-induced malignant lymphoma to other strains of mice and at fine structure examination for the presence of virus particles. Also attempts should be made to elucidate the precise mechanisms of the effects of fractionation of MNUA on the incidence of malignant lymphoma. This would involve studying the sequential histological changes, immunological suppression and other changes occurring during the genesis of malignant lymphoma induced by fractionated doses of

MNUA. The observations made in the study of serial transplantation of thymus cells during the genesis of DMBA-induced malignant lymphoma should be extended and confirmed by injecting various numbers of full-fledged neoplastic cells mixed with various numbers of normal thymus cells.

SUMMARY

The pathogenesis of chemically-induced malignant lymphoma in mice was studied from three points of view: the morphological aspects, the dose-response relationships and the transplantability of thymus cells. It was shown by these studies that:

1. All the tissues of the lymphoreticular system atrophy and regenerate during the development of MNUA-induced malignant lymphoma.
2. The thymus is the site of origin of the MNUA-induced malignant lymphoma.
3. The other tissues of the lymphoreticular system and the parenchymatous organs are sites of metastasis rather than foci of origin of malignant lymphoma.
4. There are similarities in the genesis and behaviour of MNUA-induced lymphoma and of virus-induced lymphomas.
5. A reduction of early mortality of treated mice, an enhancement of incidence and a shortening of latent period of malignant lymphoma are the main effects of fractionated doses of MNUA.

6. During the development of DMBA-induced malignant lymphoma the thymus cells become transplantable at 35 days after treatment with DMBA.
7. The malignant lymphomas occurring after transplantation of early neoplastic cells of the thymus of DMBA-treated mice show exclusive or predominant involvement of the thymus with absence of bone marrow involvement.
8. The malignant lymphomas occurring after transplantation of full-fledged neoplastic cells of DMBA-induced thymic lymphomas show widespread involvement of the lymphoreticular system with a leukaemic blood picture.

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APPENDIX

Method of sex diagnosis by karyotyping

Chromosome preparations of thymus and bone marrow of normal adult male and female mice of the CFW/D strain were made by the flame-drying technique described by Bunker (1965). It has been previously demonstrated in this laboratory that male and female cells can be identified from the karyotypes of thymus in the normal mice of CFW/D strain (P. O. Ottonen, unpublished data). Similar observations have been made in other Swiss derived strains by Stich and Hsu (1960). It has been further shown that sex diagnosis can also be made from karyotypes prepared from DMBA-induced primary thymic lymphomas having abnormal number of chromosomes (P. O. Ottonen, unpublished data). The diagnosis of sex in somatic cells of mice is based on the study of the shortest chromosomes. Female cells contain two short chromosomes of equal size which are significantly shorter than the second smallest homologous chromosome pair. Male cells contain three small chromosomes, two of them being the smallest autosomes and one representing the Y chromosome (Plates 24 and 25). Distinction between the

Y chromosome and the smallest homologous pair was not attempted and the sex diagnosis depended exclusively on the presence of three small chromosomes in male cells and two in female cells. An over contraction of the chromosomes due to prolonged treatment with colcemid was avoided. The sex diagnosis was made only on those metaphase plates showing the smallest autosomes distinctly shorter than the second smallest autosome pair. Broken cells with lost chromosomes were discarded.

Slides of chromosome preparation of thymus and bone marrow from mice of known sex were first studied to become familiar with the differences in the male and female karyotypes. This was followed by a blind study of attempting to make a sex diagnosis from the karyotypes prepared from animals whose sex was kept unknown at the time of examination of the slides. Through experience accuracy to the extent of 99% was reached in making a sex diagnosis from the karyotypes of thymus and bone marrow of normal animals.

The karyotypes of the thymic lymphomas occurring after transplantation of early and full-fledged neoplastic cells of the thymus of DMBA treated mice were examined without the knowledge of the sex of the donor or the recipient. At least 25 metaphase plates showing the smallest autosomes distinctly shorter than the second smallest autosome pair were examined from each animal with

transplanted lymphoma. The sex diagnosis made by examination of karyotypes was later checked against the records and was found to be in agreement in all the animals.